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Analysis of Ki-67 and Bcl-2 protein expression in normal colorectal mucosa of women with breast cancer

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ABSTRACT

The aim of this study was to evaluate Ki-67 and Bcl-2 protein expression in the normal colorectal mucosa adjacent to adenomatous polyps in women with breast cancer. A cross-sectional, controlled study was conducted in 35 women with and without breast cancer who had adenomatous colorectal polyps. The patients were divided into two groups: Group A (a control group of women without breast cancer, $n = 18$) and Group B (a study group of women with breast cancer, $n = 17$). A sample of normal colonic mucosa was collected at a distance of 5 cm from the polypoid lesion to evaluate immunohistochemical expression of the Ki-67 and Bcl-2 proteins. Student's *t*-test and the chi-square test were used to analyse Ki-67 and Bcl-2 expression, respectively. Statistical significance was established at $p < 0.05$. The mean percentage of Ki-67-stained nuclei in Groups A and B was 25.12 ± 2.08 and 41.50 ± 1.85, respectively ($p < 0.001$), whereas the percentage of cases with cells expressing Bcl-2 in Groups A and B was 17.6% and 82.4%, respectively ($p < 0.003$). In the present study, greater proliferative activity and greater expression of the antiapoptotic protein Bcl-2 was found in the normal colorectal mucosa of women with breast cancer.

1. Introduction

Over the past few decades, there has been a considerable increase in the survival of women with breast cancer, principally due to early diagnosis and the growing use of adjuvant therapies.[1,2] Nevertheless, a large number of women who survive breast cancer go on to develop a new primary cancer.[3,4] Knowledge with respect to the risk of women with breast cancer of developing a second primary cancer is important not only in view of the possibility that these neoplasias may share a common aetiology but also because of the possibility of establishing strategies for prevention and early diagnosis.[5]

With the exception of a contralateral breast tumour, the most common second primary cancer in women who survive breast cancer is colorectal cancer.[6] These women have an increased risk not only of carcinomas but also of developing colorectal adenomas.[7] Population-based cohort studies suggest that the risk of colorectal cancer is high, even in female relatives of women with breast cancer compared to the general population.[8]

Some studies have shown an increase in the proliferative activity of normal colonic mucosa in patients with colonic disease such as adenomas or carcinomas compared to control patients.[9,10] The imbalance between cell proliferation and programmed cell death at colonic crypt level is a key phenomenon in the development of colorectal cancer.[7–9] Evaluation of the expression of biological markers of proliferation and apoptosis in normal colonic mucosa may become an

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important strategy for detecting a risk of malignisation.[10] In this respect, Ki-67 and Bcl-2 are becoming the most commonly used markers in the evaluation of cell proliferation and apoptosis, respectively.[10–12]

There is a paucity of studies in the medical literature evaluating cell kinetics in the normal colorectal mucosa of women with breast cancer. To the best of our knowledge, only one study in the literature has evaluated proliferative activity in the normal rectal mucosa of women with breast cancer, using tritiated thymidine-labelling[13]; however, apoptotic activity was not evaluated, which led us to design the present study.

2. Patients and methods

2.1. Patients

This study was approved by the Internal Review Board of the Federal University of Piauí and all patients gave their signed informed consent prior to study initiation. Two hundred ninety-four women were included in this study between October 2007 and December 2008. Sixty-four patients with newly-diagnosed breast cancer (study group) were recruited from the mastology clinic of the *Getúlio Vargas* Hospital, Federal University of Piauí, whereas 230 patients without breast cancer (control group) were recruited from the proctology clinic of the General Surgery Department in the same institute. All patients with breast cancer were volunteers for a colonoscopy exam in the context of the research project, whereas the patients of the control group were submitted to the colonoscopy exam due to gastrointestinal symptoms. Of the 64 patients with breast cancer, 20 had colorectal polyps and of these, 3 were excluded because of technical difficulties, remaining 17 patients for the study of biomarkers. Of the 230 women without breast cancer, 23 had colorectal polyps and of these, 5 were excluded because of technical difficulties, remaining 18 women for the study of markers. The women were simply recruited from the two sources and at the end of a 14-month period, 20 or more women were found to have colorectal polyps in each group. None of the woman in the study group had BRCA-associated breast cancers. Breast cancer patients with a history of prior treatment or in 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 patients without breast cancer had been submitted to imaging tests (mammography and ultrasonography) with negative results for malignancy. None of the patients enrolled in this study had family history of breast cancer or any prior history of colonoscopy, gastrointestinal surgery or family history of gastrointestinal cancer.

2.2. 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, $n = 18$) and Group B (breast cancer, study group, $n = 17$). These groups were considered homogenous with respect to age, age at menarche, parity and menopausal status (Table 1).

Table 1 – Characteristics of the patients.

	A (control) $n = 18$	B (study) $n = 17$	p-Value
Age (years)			
Mean	53.56	54.53	0.810
Menarche age (years)			
Mean	13.77	13.64	0.799
Delivery number			
Mean	3.72	4.23	0.640
Menopausal status			
Yes	15 (83%)	13 (76%)	0.691
No	3 (17%)	4 (24%)	

2.3. 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 exam, the fasted patients ingested 500 ml of 20% Mannitol and the exam was performed four hours later using an Olympus CF-100 colonoscope (Olympus Optical Co. Ltd., Tokyo, Japan). In all cases, colonoscopy was performed under anaesthetic sedation and by the same physician, who was blinded with respect to the identification of the cases. When polyps were removed, a biopsy of the colorectal mucosa was taken at a distance of 5 cm from the polypoid lesion. The surgical specimens were fixed in buffered formalin for 12–24 h and subsequently submitted to histopathological analysis using a standardised procedure to confirm the diagnosis of an adenomatous polyp and of normal colorectal mucosa.

2.4. Immunohistochemistry for Ki-67 and Bcl-2

For the immunohistochemical evaluation of Ki-67 and Bcl-2 protein expression, the samples of normal colorectal mucosa fixed in buffered formalin were cut into 3-μm-thick sections. Next, the sections were deparaffinised in xylol for 5 min, dehydrated in absolute ethanol and washed in buffered saline solution at pH 7.4 for 5 min. Subsequently, the sections were treated for 5 min with 3% hydrogen peroxide (H_2O_2) diluted in buffered solution to block endogenous peroxide. 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 overnight at 4–8 °C with primary mouse anti-Ki-67 monoclonal antibody (clone MIB1, Ref. M7240, Dako, Carpinteria, USA/1:4800) and with mouse anti-Bcl-2 monoclonal antibody (clone 124, Ref. M0887, Dako, Carpinteria, USA/1:2000). The slides were then washed with PBS-Tween and instilled with secondary reagent (anti-mouse BA 2000, Vector, Burlingame, USA), incubated for 60 min at room temperature, washed again in PBS-Tween, and instilled with the ABC Elite detection system (PK 6100, Vector, Burlingame,

USA), incubated for 45 min at room temperature, washed once again with PBS-Tween, instilled with DAB (Diaminobenzidine tetrahydrochloride, Ref. D5637, Sigma, St. Louis, USA) and incubated for five minutes. Finally, the slides were washed with distilled water, counterstained with haematoxylin, 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 colouring of the nucleus and cytoplasm, respectively.

2.5. *Quantitative method*

Quantification was carried out by two observers who were blinded with respect to the patients' identity and had no previous knowledge of any of the cases. It was performed using a light microscope (Nikon Eclipse E-400, optical microscope, Tokyo, Japan) connected to a colour video-camera (Samsung digital camera CHC-370 N, Seoul, Korea) which captured the image and transmitted it to a computer equipped with the Imagelab® software programme, version 2.3, developed by Softium Informática Ltda. (São Paulo, Brazil) for image analysis. For Ki-67 expression, a minimum of 400 cells were counted on each slide using magnification of 400×, whether 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, 7–9 (mean 8) colonic crypts 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 (labelling index). Bcl-2 immunoreaction was evaluated semi-quantitatively according to the criteria established by van Slooten and colleagues[14] taking the following parameters into consideration: intensity of cell staining (*I*) and the fraction of stained cells (*F*). The intensity of cell staining was classified as: 0 (negative), 1 (weakly stained), 2 (moderately stained) and 3 (strongly stained). The fraction of stained cells was classified as: I (0–25%), II (25–75%) or III (75–100%). 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 $\geqslant 3$ were classified as positive for Bcl-2. In all cases, brownish staining in the cytoplasm was adopted as the standard for positivity.[14,15]

2.6. *Statistical analysis*

Student's *t*-test was used to verify homogeneity between the two groups with respect to the patients' age and age at menarche,[16] and Fisher's exact test was used to evaluate parity and menopausal status.[17] Student's *t*-test was used to compare the means of the percentages of Ki-67-stained nuclei between the two groups and comparison of the proportions of cases with cells expressing Bcl-2 in the colorectal mucosa was performed using the chi-square test.[18] Significance was established at $p < 0.05$ throughout the study.

3. Results

Under light microscopy, there was a greater concentration of Ki-67-stained nuclei in the crypts of the normal colorectal

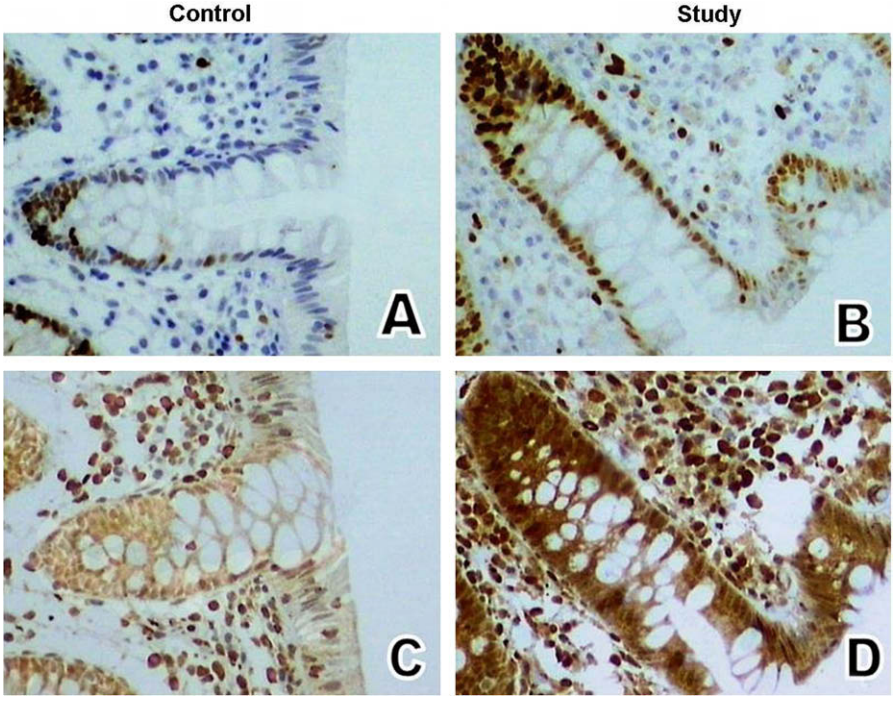

Fig. 1 – Photomicrographies of histological sections of normal colorectal mucosa. Note the lower concentration of Ki-67 stained nuclei in the cells in the normal colorectal mucosa of the control patient (A) compared to the higher concentration of this marker in the colorectal mucosa of the patient with breast cancer (B). Note the negative expression of Bcl-2 protein in the colorectal mucosa of the control patient above (C) compared to the proportion of cells with cytoplasms intensely stained brown by Bcl-2 in the colorectal mucosa of the patient with breast cancer (D). Original magnification 200×.

Table 2 – Mean percentage of Ki-67 stained nuclei in the normal colorectal mucosa of patients in the control (A) and study (B) groups.

Group	*n*	Mean	SE Mean	Minimum	Median	Maximum
A	18	25.12	2.08	13.13	23.88	43.28
B	17	41.50[a]	1.85	27.62	42.67	57.98

a The difference between the two groups was statistically significant ($p < 0.001$).

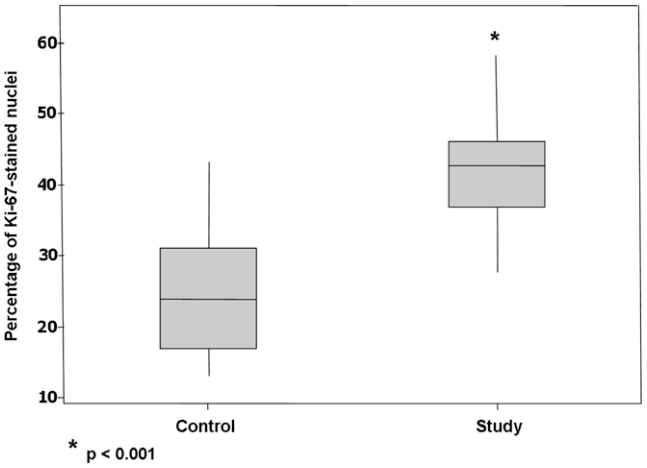

Fig. 2 – Boxplot of the percentage of cells with Ki-67 stained nuclei in the normal colorectal mucosa of the patients in the control (A) and study (B) groups.

Table 3 – Percentage of cases with cells expressing Bcl-2 in the normal colorectal mucosa of patients in Groups A (control) and B (study).

Group	Negative *n* (%)	Positive *n* (%)	Total *n* (%)
A	12 (66.7)	6 (33.3)	18 (100.0)
B	3 (17.6)	14 (82.4)[a]	17 (100.0)
Total	15 (45.4)	20 (55.6)	35 (100.0)

a The difference between the two groups was statistically significant ($p < 0.003$).

mucosa of patients with breast cancer (Group B) compared to those of Group A (controls). There were also more cases with a positive expression for Bcl-2 protein in the colorectal mucosa of women in the study group as shown by a greater fraction of cells with intensely stained cytoplasm compared to the control group (Fig. 1). The mean percentage of Ki-67-stained nuclei was 25.12 ± 2.08 and 41.50 ± 1.85 in the crypts of the colorectal mucosa of patients in Groups A (control) and B (study), respectively ($p < 0.003$) (Table 2 and Fig. 2). With respect to Bcl-2 antigen expression, the number of cases with positive expression for Bcl-2 in the crypts of the colorectal mucosa of patients in Groups A and B was 6/18 (33.3%) and 14/17 (82.4%), respectively ($p < 0.001$) (Table 3).

4. Discussion

The findings of various population-based cohort studies suggest an increased risk of colorectal cancer in women with breast cancer compared to women in the general population,[3] and this risk varies, according to some studies, from marginally increased to higher relative risks.[19,20] All these studies are epidemiological and the subsequent risk of colorectal cancer in these patients remains unclear.[3] On the other hand, some investigators have shown that colorectal epithelial cell kinetics change in patients at increased risk for colon cancer.[13] However, there is a paucity of studies evaluating the expression of proteins related to proliferative and apoptotic activity in the normal colonic mucosa of women with breast cancer, despite some studies have shown an increase in the prevalence of colorectal adenomas in these patients.[7,21,22]

Ochsenkühn and colleagues,[7] in a cross-sectional study, showed that women with primary breast cancer had a higher risk of colorectal adenomas than matched controls with an odds ratio of 1.7. Bremond and colleagues[21] showed that women with breast cancer had a 2.5-folder higher incidence of colorectal adenomas than the control group. Rozen and colleagues[22] found colorectal adenomas to be 2.7–3.0 more common in women with breast cancer than in controls, which is in agreement with the rate of colorectal adenomas found in patients with breast cancer in this study, corroborating with the importance of the analysis of cell proliferation and apoptosis markers in the normal colorectal mucosa adjacent to adenomas of patients with breast cancer.

In the present study, Ki-67 protein expression was significantly higher in the crypts of the normal colorectal mucosa of women with breast cancer compared to that of control women. Likewise, the number of cases with positive expression of the antiapoptotic protein Bcl-2 was significantly higher in the crypts of the colorectal mucosa of women with breast cancer compared to the control group. These findings are in agreement with the only study in the literature in which, although the investigators failed to include an evaluation of apoptotic activity, proliferative activity in the normal rectal mucosa of women with breast cancer was assessed using tritiated thymidine-labelling.[13] Patients with history of any treatment for the breast cancer were excluded from the study, therefore none of them was receiving tamoxifen, since it has been shown in animal models that selective oestrogen receptor modulators may decrease colonic cell growth.[23]

Colorectal epithelial cell proliferation is greater in individuals with a higher risk of colorectal neoplasias and consequently has been proposed as a biomarker for the prevention of colorectal cancer.[24] However, the ability of this measurement of proliferative activity to predict future adenomas or colorectal cancer remains unclear[3,25] and requires further studies. Ki-67 protein was used in the present study in view of its sensitivity as a marker of cell proliferation since this protein is expressed in all the phases of the cell cycle except in the resting phase (G0).[11,26,27] Moreover, an increase in

its expression has been associated with greater aggressivity of neoplasias.[10–12,28]

To calculate the Ki-67 labelling index (LI) of normal colorectal mucosa, biopsies were obtained at the time of excision of adenomatous colorectal polyps at a distance of 5 cm from the polypoid lesion. Some investigators have divided colonic crypts into two or more parts for the evaluation of immunoreactivity, taking into consideration that it is in the lower third of the crypt that the germinative cells are found and it is in these cells that proliferative activity is greatest.[28] Nevertheless, in the present study, in order to evaluate all proliferative activity, the entire extension of colonic crypts (a mean of 7–9 crypts for each case) was studied.

With respect to Bcl-2, this protooncogene encodes the protein that protects cells from programmed cell death, this protein being expressed in the proliferative compartment of several tissues, including normal colonic crypts.[29] Bosari and colleagues[30] showed that the normal colorectal mucosa of patients who have premalignant or malignant colonic lesions positively expresses Bcl-2; however, the majority of carcinomas do not. Likewise, Flohil and colleagues[29] also demonstrated an increase in Bcl-2 expression in adenomatous colorectal polyps but not in carcinomas, suggesting that the Bcl-2 oncoprotein may play a role in colorectal tumourigenesis, probably in the early phases of the adenoma–carcinoma sequence.[29,30] These studies are in agreement with our findings, showing a greater expression of the antiapoptotic protein Bcl-2 in the normal colonic mucosa of women with breast cancer, who are known to have a greater risk of developing colorectal adenomas and carcinomas.

To the best of our knowledge, this is the first study to evaluate Ki-67 and Bcl-2 expression in the normal colorectal mucosa of women with breast cancer. Although further studies are required, the present findings show that in relation to the controls women with breast cancer had greater proliferative activity and a greater expression of the antiapoptotic protein Bcl-2 in normal colorectal mucosa, thereby possibly suggesting a higher risk for the development of premalignant and malignant lesions, and emphasising the importance of the use of routine colonoscopy in these patients to screen for such lesions.

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Conflict of interest statement

None declared.

REFERENCES

1. Sant M, Capocaccia R, Coleman MP, et alEUROCARE Working Group. Cancer survival increases in Europe, but international differences remain wide. *Eur J Cancer* 2001;**37**:1659–67.
2. Clegg LX, Li FP, Hankey BF, Chu K, Edwards BK. Cancer survival among US whites and minorities: a SEER (Surveillance, Epidemiology, and End Results) Program population-based study. *Arch Intern Med* 2002;**162**:1985–93.
3. Kmet LM, Cook LS, Weiss NS, Schwartz SM, White E. Risk factors for colorectal cancer following breast cancer. *Breast Cancer Res Treat* 2003;**79**:143–7.
4. Schaapveld M, Visser O, Louwman MJ, et al. Risk of new primary nonbreast cancers after breast cancer treatment: a Dutch population-based study. *J Clin Oncol* 2008;**26**:1239–46.
5. Mellemkjaer L, Friis S, Olsen JH, et al. Risk of second cancer among women with breast cancer. *Int J Cancer* 2006;**118**:2285–92.
6. Khalil S, Ceccarelli F. Colorectal cancer after breast cancer: magnitude of risk in clinical practice and in the literature. *Tumori* 2009;**95**:28–31.
7. Ochsenkühn T, Bayerdörffer E, Meining A, et al. Increased prevalence of colorectal adenomas in women with breast cancer. *Digestion* 2005;**72**:150–5.
8. Trentham-Dietz A, Newcomb PA, Nichols HB, et al. Breast cancer risk factors and second primary malignancies among women with breast cancer. *Breast Cancer Res Treat* 2007;**105**:195–207.
9. Sandler RS, Baron JA, Tosteson TD, Mandel JS, Haile RW. Rectal mucosal proliferation and risk of colorectal adenomas: results from a randomized controlled trial. *Cancer Epidemiol Biomarkers Prev* 2000;**9**:653–6.
10. Akedo I, Ishikawa H, Ioka T, et al. Evaluation of epithelial cell proliferation rate in normal-appearing colonic mucosa as a high-risk marker for colorectal cancer. *Cancer Epidemiol Biomarkers Prev* 2001;**10**:925–30.
11. da Silva BB, Lopes IM, Gebrim LH. Effects of raloxifene on normal breast tissue from premenopausal women. *Breast Cancer Res Treat* 2006;**95**:99–103.
12. da Silva BB, Pires CG, Dos Santos AR, et al. Effects of raloxifene on Ki-67 and CD34 antigen expression in breast cancer. *Gynecol Obstet Invest* 2009;**67**:103–8.
13. Rozen P, Fireman Z, Fine N, Chetrit A, Zajicek G. Rectal epithelial proliferation in women recovered from breast cancer. *Cancer Lett* 1990;**55**:189–94.
14. van Slooten HJ, Clahsen PC, van Dierendonck JH, et al. Expression of Bcl-2 in node-negative breast cancer is associated with various prognostic factors, but does not predict response to one course of perioperative chemotherapy. *Br J Cancer* 1996;**74**:78–85.
15. dos Santos LG, Lopes-Costa PV, dos Santos AR, Facina G, da Silva BB. Bcl-2 oncogene expression in estrogen receptor-positive and negative breast carcinoma. *Eur J Gynaecol Oncol* 2008;**29**:459–61.
16. Bussab W, Morettin PA. *Estatística básica*. São Paulo: Editora Saraiva; 2002.
17. Agresti A. *Categorical data analysis*. 2nd ed. New York: John Wiley and Sons Inc.; 2002.
18. McCullogh P, Nelder JA. *Generalized linear models*. 2nd ed. London: Chapman and Hill; 1989.
19. Harvey EB, Brinton LA. Second cancer following cancer of the breast in Connecticut, 1935–82. *Natl Cancer Inst Monogr* 1985;**68**:99–112.
20. Buiatti E, Crocetti E, Acciai S, et al. Incidence of second primary cancers in three Italian population-based cancer registries. *Eur J Cancer* 1997;**33**:1829–34.
21. Bremond A, Collet P, Lambert R, Martin JL. Breast cancer and polyps of the colon: a case control study. *Cancer* 1984;**54**:2568–70.
22. Rozen P, Fireman Z, Figer A, Ron E. Colorectal tumor screening in women with a past history of breast, uterine, or ovarian malignancies. *Cancer* 1986;**57**:1235–9.
23. Janakiram NB, Steele VE, Rao CV. Estrogen-beta as a potential target for colon cancer prevention: chemoprevention of

azoxymethane-induced colon carcinogenesis by raloxifene in F344 rats. *Cancer Prev Res (Phila Pa)* 2009;**2**:52–9.
24. Lipkin M, Higgins P. Biological markers of cell proliferation and differentiation in human gastrointestinal diseases. *Adv Cancer Res* 1988;**50**:1–24.
25. Rooney PS, Clarke PA, Gifford KA, Hardcastle JD, Armitage NC. The identification of high and low risk groups for colorectal cancer using rectal mucosal crypt cell production rate (CCPR). *Br J Cancer* 1993;**68**:172–5.
26. Gerdes J, Lemke H, Baisch H, et al. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *J Immunol* 1984;**133**:1710–5.
27. da Silva BB, dos Santos AR, Pires CG, Lopes-Costa PV. Effect of raloxifene on vascular endothelial growth factor expression in breast carcinomas of postmenopausal women. *Cell Prolif* 2009;**42**:506–10.
28. Risio M, Coverlizza S, Ferrari A, Candelaresi GL, Rossini FP. Immunohistochemical study of epithelial cell proliferation in hyperplastic polyps, adenomas, and adenocarcinomas of the large bowel. *Gastroenterology* 1988;**94**:899–906.
29. Flohil CC, Janssen PA, Bosman FT. Expression of Bcl-2 protein in hyperplastic polyps, adenomas, and carcinomas of the colon. *J Pathol* 1996;**178**:393–7.
30. Bosari S, Moneghini L, Graziani D, et al. Bcl-2 oncoprotein in colorectal hyperplastic polyps, adenomas, and adenocarcinomas. *Hum Pathol* 1995;**26**:534–40.