ARTIGO ORIGINAL

Expression of BCL-2 and BAX proteins in breast cancer after *in vivo* tamoxifen administration

Expressão das proteínas BCL-2 e BAX no câncer de mama após administração in vivo de tamoxifeno

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Keywords

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ABSTRACT

Despite the presence of estrogen receptor in breast cancer cells, some patients do not show benefits on their treatment with tamoxifen, which is a medication that interferes on cell cycle promoting apoptosis. Since BCL-2 and BAX proteins are directly linked to this process, it is important to understand which pathways for cell death are related to and interfered by tamoxifen. Paired samples of breast cancer tumors, which were previously obtained before and after tamoxifen therapy were randomly divided in the Control and Treated Patients Groups. The Treated Group received tamoxifen for 14 days (20 mg/day). The immunohistochemical identification of BAX and BCL-2 expression was analyzed in a semi-quantitative scale considering the number of positive cells and intensity of staining. The scores of each reaction were compared pre and post–treatment and to the Control Group. Over the 25 patients studied, considering no exposure to the drug, there were 36 (9/25) and 72% (18/25) of positive cases for BCL-2 and BAX, respectively. This study showed no significant changes over BCL-2 and BAX proteins expression after 14 days of treatment with tamoxifen ($p > 0,05$) compared to the control patients. Expression of BCL-2 and BAX proteins did not suffer statistically significant changes after a 14-day exposure to tamoxifen. This is one of a few prospective randomized double-blind studies about *in vivo* effects of tamoxifen in apoptosis.

Descritores

Câncer de mama
Imunoistoquímica
Apoptose
Ciclo celular
Tamoxifeno
Proteína BAX associada à BCL-2

RESUMO

Apesar de apresentarem receptores estrogênicos nas células tumorais, algumas pacientes com câncer de mama não se beneficiam do tratamento com tamoxifeno, medicamento conhecido por interferir no ciclo celular e promover apoptose. Sabendo-se que as proteínas BCL-2 e BAX estão diretamente relacionadas a este processo, é importante compreender quais os caminhos que levam à morte celular que sofrem interferência do tamoxifeno. Amostras pareadas de câncer de mama foram obtidas antes e após tratamento de pacientes previamente, assim randomizadas: grupo controle e grupo de tratamento com tamoxifeno. O grupo de tratamento recebeu tamoxifeno (20 mg/dia) por 14 dias. A identificação imunoistoquímica da expressão de BAX e BCL-2 foi analisada de forma semiquantitativa, considerando o número de células coradas e a intensidade da coloração. Os escores de cada reação foram comparados pré e pós-tratamento, e também em relação ao grupo controle. Das 25 pacientes estudadas, considerando nenhuma exposição ao medicamento, foram encontrados 36 (9/25) e 72% (18/25) de casos positivos para BCL-2 e BAX, respectivamente. Este estudo não encontrou mudanças significativas sobre a expressão das proteínas BCL-2 e BAX, após 14 dias de tratamento com tamoxifeno ($p>0,05$), comparado ao grupo controle. A expressão das proteínas BCL-2 e BAX também não sofreu mudanças significativas após 14 dias de exposição ao tamoxifeno. Este é um dos poucos trabalhos prospectivos randomizados de estudo in vivo dos efeitos do tamoxifeno na apoptose.

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Introduction

Tamoxifen, a widely used drug for the treatment of estrogen receptor-positive (RE+) breast cancer, significantly reduces the incidence of new tumors and the local recurrence of this neoplasia. The treatment increases the disease-free interval, as well as diminishes the appearance of tumors in the contralateral breast[1-3]. This drug also has a potential effect in reducing the incidence of noninvasive carcinomas (*in situ*) of up to 50%[4].

This drug mainly acts in the control of the cellular cycle, either through an antiproliferative effect (diminishing the contingent of cells to enter in cellular division) and/or inducing apoptosis.

However, the real importance of these two mechanisms in the clinical effectiveness of tamoxifen remains only partially known[5], and the extension of the consequences of the estrogenic blockade action in tumoral cells was not totally elucidated. Apoptosis or programmed cellular death is a physiological process to eliminate senescent or damaged cells. The mechanism is initiated or interrupted under action of specific proteins, it is positively or negatively regulated through controlling genes products[6]. BCL-2 and BAX are two proteins involved in the mitochondrial control of the apoptosis complex mechanism. They have very distinct actions, with opposite effects. BCL-2 (with other members of the family BCL-2) has an anti-apoptotic effect and BAX works as an opponent, with the ability of inducing cell death[7,8]. The relationship between BCL-2/BAX functions and the anti-estrogenic effect caused by tamoxifen treatment are not yet totally elucidated[5,9-12].

It would be of great interest to inquire if the BCL-2 and BAX cellular expression is modified after the introduction of tamoxifen therapy. The aim of this study was to analyze the immunohistochemical expression of BCL-2 and BAX in patients with breast invasive carcinoma, previously known as positive hormonal receptor expression, treated with tamoxifen for 14 days and then submitted to surgery, and also to compare them to a Control Group that did not receive tamoxifen therapy.

Materials and methods

Sample

Archival paraffin-embedded tumoral samples were obtained from patients with breast carcinoma under medical care at our university hospital, from February 2000 to December 2002, according to a protocol previously approved by the Human Investigation Committee. All participants provided written informed consent for this study. All of them had positive expression for estrogen and/or progesterone receptors (ER+/RP+) and had been submitted to definitive surgical treatment (radical mastectomy or quadrantectomy with axillary dissection) in an average period of four weeks after the first intervention (incisional biopsy).

Patients with endocrine diseases, hormonal therapy users or the ones in the pregnant-puerperal cycle in the 12 months that preceded the diagnosis were excluded, as the patients with negative expression for estrogen and/or progesterone receptors. Women with thromboembolism history were excluded, as well as patients that had previously been submitted to any treatment for breast cancer (surgery, radio, and/or chemotherapy).

Patients were randomly distributed into two groups: one received tamoxifen for 14 days (14 patients) and the other one (Control) did not receive the medication (15 patients).

The first tumoral sample was obtained from diagnostic incisional biopsies executed in the outpatient service, using local anesthetic with no epinephrine (2% lidocaine). Only one fragment was obtained.

Patients selected to receive the medication started to take it 14 days prior to definitive surgery.

A second fragment from the neoplastic tissue was obtained during definitive surgery, under general anesthesia, from patients who received tamoxifen and from those who did not. This second fragment was submitted to the same paraffin blocking technique used when the initial samples were obtained.

Histology and immunohistochemistry

Formalin-fixed, paraffin-embedded tissue samples of 14 treated and 15 nontreated with tamoxifen patients were retrieved from the pathology files of UNIFESP-EPM. For each patient, another biopsy was obtained 14 days after the treatment as previously stated. Samples were immersed in 10% neutral-buffered formalin and embedded in paraffin. Fragments were available in archives obtained from the initial biopsy and at definitive surgery.

Tumor pairs were cut into 4 µm sections and mounted onto lysine-coated slides, stained in a routine manner with hematoxylin-eosin, and the presence of carcinoma in samples was confirmed according to the World Health Organization criteria[13].

The BCL-2 and BAX expressions were identified in breast carcinoma cells through the immunohistochemical method of the streptavidin-biotin-peroxidase, according to the technique that will be further described.

Paraffin was removed from the slides by heating them at 60 °C for 10 minutes, followed by three washes in xylene and rinsed in graded alcohols. After being deparaffinized and rehydrated, tissue sections were submitted to heat-induced antigen retrieval by pressure cooking in 10 mMol/L citrate buffer (pH = 6.0). We blocked the endogenous peroxidase with hydrogen peroxide (H_2O_2) at 3% in four immersions during five minutes each, followed by washing with distilled water and in phosphate buffered saline (PBS) of 0.01M, pH of 7.4.

Slides were incubated with the respective primary antibodies BCL-2 (DAKO, code M0887, clone 124, Lot 011-401), in the 1:50 dilution and BAX (DAKO, code A3533, policlonal, Lot 057Ea) and 1:200 dilution. The slides were kept two hours under room temperature and then washed with PBS and pH of 7.4.

As a secondary antibody, to amplify the reaction, we used the Kit DAKO Cytomation LSAB+System-HRP (code K0690) with a 20-minute incubation, and then another 20-minute incubation with the streptavidin-peroxidase complex, under room temperature and washed in PBS and pH of 7.4.

After incubation with diaminobenzidine for five minutes at 37 °C, sections were counter stained with Harris hematoxylin for two minutes.

Sections of a lymph node that was previously known as positive were used as positive staining control, and as negative control, sections incubated with PBS solution were used instead of the primary antibody. We considered as positive all reactions with brown colored cells.

Quantization and scoring of BAX and BCL-2 expressing cells

All slides were reviewed by two investigators and scored, based on Allread's criteria in a semi-quantitative manner with two parameters: number of stained cells and intensity of color staining[14,15]. Regarding the intensity of staining, we used the scores ranging from zero to three. When considering the total amount or fraction of stained cells on each slide, a scale from zero to five was adopted. These parameters were used for each immunoreaction. The sum of these two partial scores resulted in a final score that could be zero, if no cell was stained, and could range from two to eight in positive cases, which means that we considered positive all cases with a final score different from zero.

Statistical analysis

BAX and BCL-2 immunoreactions between both groups were compared, before and after treatment, and the same was done to control cases in initial biopsies and samples obtained at definitive surgery. The variability analysis test was used to verify differences in BCL-2 and BAX mean values among the Tested and Control Groups, in order to search for behavioral changes among the paired samples into two distinct moments for the same case[16].

The Student's *t*-test was applied, comparing the mean of scores for BCL-2 in control patients with those of the patients treated with tamoxifen. The same analysis was used to evaluate means of BAX scores.

Fisher's test was applied to compare differences in BCL-2 and BAX expressions (negative: score = zero; positive: score ≠ zero).

Results

Four cases were discarded due to lack of tumor representativity in the sample of the block, with fragment obtained during definitive surgery. Two of the patients belonged to the Control Group and two to the group treated with tamoxifen. Thus, 13 control patients and 12 treated patients remained in the study.

In the 25 patients included in the study, considering the initial samples from all patients, i.e., none still exposed to the medication, a 36%-positivity was identified for BCL-2 and a 72%-positivity for BAX (Table 1).

Analyzing separately the frequencies, the Control Group had 33.3% positivity for BCL-2 in the biopsy samples and 66.6% in the samples of the tumors obtained at the time of the definitive surgery (Table 1).

The frequency of BAX immunoreaction in the Control Group was 66.6% in the samples of the biopsies and 75% in the samples obtained at surgery, without any significant differences between the means for both reactions (Table 1).

In the group treated with tamoxifen, positivity of the reaction for BCL-2 occurred in 38.5% of the cases before the treatment, and in 46.2% of the cases after using medication (Table 1). In this group, the immunoreaction for BAX was also positive in 76.9% of the pre- cases and in 92.3% of the cases post-treatment (Table 1). No statistical difference was found between the frequencies of BCL-2 and BAX comparing the pre and post-treatment means.

Analysis of BCL-2 expression

In the Control Group (n = 12), eight cases were negative in the initial samples (incisional biopsies) and the four positive cases found in this group presented scores ranging from three to seven. In the surgical samples (definitive surgery), we found four negative cases and the eight positive ones presented a score range varying from five to eight.

Table 1. Immunoreaction results for BCL-2 and BAX in the Control Group (C) and in the group treated with tamoxifen (T)

BCL-2 Groups	Pre*		Post†	
	Negative	Positive	Negative	Positive
C (n = 12)	8 (66.6%)	4 (33.3%)	4 (33.3%)	8 (66.6%)
T (n = 13)	8 (61.5%)	5 (38.5%)	7 (53.8%)	6 (46.2%)
BAX Groups	Pre‡		Post§	
	Negative	Positive	Negative	Positive
C (n = 12)	4 (33.3%)	8 (66.6%)	3 (25%)	9 (75%)
T (n = 13)	3 (23.1%)	10 (76.9%)	1 (7.7%)	12 (92.3%)

*p = 1.00; †p = 0.428; ‡p = 0.673; §p = 0.322.

Thus, the profile of BCL-2 expression remained unchanged in six cases and among the six other remaining ones, five began to express BCL-2 and only one stopped expressing the protein, but these differences were not statistically significant (Table 2).

In the group of patients that received tamoxifen (n = 13), considering pre-treatment samples, eight cases were negative, and the five cases that became positive presented scores ranging from two to eight. In the post-treatment samples, seven negative cases were found and six with a positivity score ranging from three to eight.

When analyzing these results as a whole, it was observed that from the eight initial negative cases, four remained nonreactive and the other four began to express BCL-2. On the other hand, among the five previously positive cases, after treatment with tamoxifen, two cases remained positive and three cases became negative.

An overview showed that, in six cases in the Tamoxifen Group, BCL-2 expression was not significantly changed by treatment and, seven of these patients had no changes in the BCL-2 expression profile. However, comparing pre and post-treatment samples, these modifications did not show a statistical difference (Table 2).

Analysis of BAX expression

The BAX immunoreaction in the Control Group (n = 12), at the time of the initial biopsy, was positive in eight cases and negative in the four others, with a positivity score ranging from two to eight. In the tumor samples obtained from the surgical specimen (after 14 days), three cases were negative and in the nine positive cases, the score ranged from six to eight.

Therefore, the Control Group demonstrated that nine cases remained unchanged (positive/positive and negative/negative). Of the three cases that presented a modification of the BAX protein expression, two previously negative cases became positive and one negative case presented a positive immunoreaction. These changes in pattern were not statistically significant (Table 2).

In the patients from the Tamoxifen Group, in the pre-treatment biopsy, ten patients were positive for BAX and three did not express the protein. The scores of positive cases ranged from three to eight. After 14 days, 12 positive cases were found, with scores between six and eight, and only a negative case. In a case by case analysis, it is observed that after treatment with tamoxifen, the three previously negative cases became positive, and of the ten previously positive cases, only one case became negative. In other words, after using tamoxifen for 14 days, it was noted that in nine cases the expression of BAX was not significantly changed by treatment, and in three of them, there was a change in the BAX expression profile, but no statistical differences were found between the pre and post-tamoxifen samples (Table 2).

Table 2. Relationship of variations of BAX and BCL-2 immunoreactions comparing the different sampling times in the pre and post-tamoxifen sampling of treated patients

Tested proteins	NRN	PRP	NBP	PBN
BAX Control (n = 12)	2	7	2	1
BAX Treated (n = 13)	-	9	3	1
BCL-2 Control (n = 12)	3	3	5	1
BCL-2 Treated (n = 13)	4	2	4	3

NRN: negative cases that remained negative; PRP: positive cases that remained positive; NBP: negative cases that became positive; PBN: positive cases that became negative.

Discussion

It is still not known the action mechanism of antiestrogen drugs. The intention of this study was to see whether there is a relationship between *in vivo* exposure of tumor cells to tamoxifen and some cell death regulators, which could signal the prediction of the response.

Tamoxifen can trigger apoptosis in tumor cells from the beginning of therapy, and the number of apoptotic corpuscles increases 48 hours after the medication begins to be used[17]. This study analyzed the relationship between the expression of BAX and BCL-2 proteins before and after a 14-day exposure to tamoxifen, seeking to correlate possible changes compared to the Control Group.

When comparing the results of this study to those in the literature, it is difficult to directly confront the data. The reason is that comparisons of work published so far are methodologically limited (Table 3)[11,18-24].

In the 25 cases in this study, no distinction was made for axillary status nor histological type, since the used sample was relatively small. A study by Yang et al. did not find a relationship between the expression of BAX and BCL-2 and age, menopausal status, tumor size, histological degree, or axillary lymph node status[25].

This study enabled to observe strong BAX reactivity in the samples that presented an *in situ* component (four cases with *in situ* components and all with a strongly positive reaction for BAX and BCL-2), such fact agrees with the results of Krajewski et al[26], who identified 98% positivity for BAX in samples of intraductal carcinoma.

As to the BAX and BCL-2 frequency in the tumors, in this study, an initial analysis of the positivity of these proteins

Table 3. Studies including immunohistochemical evaluation of BCL-2 and BAX

Author	Positivity criteria	Percentage of positivity*
Hurlimann et al.[18]	> 30% stained cells	48%
Silvestrini et al.[19]	> 40% stained cells	21%
Elledge et al.[20]	> 10% stained cells	58%
Veronese et al.[21]	> 20% stained cells	67%; 45 to 55% f/ BAX
Wu et al.[22]	> 25% stained cells	65.9%; 64.8% f/ BAX
Kymionis et al.[23]	†	46%; 49% f/ BAX
Tran and Lawson[24]	More intensive staining than negative control	31 to 75%
Buchholz et al.[11]	0-5%, neg; 5-25%,+; 25-50%,++; > 50%,+++	†

* values for BCL-2; when BAX is included in the study, this is specified; †values or parameters not referred in the text.

throughout the sample was performed, i.e., in the 25 cases selected, they happened before beginning exposure to tamoxifen in the Treatment Group. Thus, 72% immunoreaction positivity was found for BAX and 36% for BCL-2, which agrees with the current publications on the subject[9,27-29].

However, in order to calculate the variation of means, the groups were evaluated apart, the frequency of each protein being evaluated for controls and treated before and after 14 days.

The Control Group did not present a significant difference in the BAX immunoreaction in the biopsy samples compared with the samples obtained during the definitive surgery. Likewise, the comparison of immunoreaction means of BCL-2 scores between biopsies and surgical samples did not present a statistically significant variation. The absence of this variation was expected, since this group could simply be subject to biological changes that are natural for the tumor.

On the other hand, the group that was treated for 14 days did not show a significant variance for the antibodies tested. Comparing the means of the immunoreaction of BAX scores pre and post-treatment, we found $p = 0.625$, which reject the assumption that tamoxifen might interfere in the expression of this pro-apoptotic protein.

The same occurred with the BCL-2 immunoreaction, in which the comparison of means before and after exposure to tamoxifen found a value of $p = 1.00$. This supports the findings of Farczádi et al.[30], who did not find changes in the expression of BCL-2 after seven days of treatment with tamoxifen under the same dose used here.

The finding that neither BAX nor BCL-2 underwent any significant changes after treatment with tamoxifen indicates that these proteins are expressed or inhibited by the same mechanism, even if they are a result of the same cellular process.

Although the groups are homogeneous, it was noticed that they presented very high standard deviations, showing that few patients had a high positivity of a given protein, while other patients did not show immunoreaction. It was also observed that, in the Control Group, the expression of BAX and BCL-2 did not appear to be constant over time.

Even without exposure to medication, changes were seen in the scores of the respective immunoreactions. Some that were previously negative became positive and others whose scores were previously positive became negative. However, there was no technical bias, since all samples were processed and submitted to reactions under the same conditions.

Other authors used tumor cell cultures to evaluate effects of tamoxifen on apoptosis, observing a reduction of BCL-2 positivity after 48 hours of exposure to the drug. Furthermore, they found a concomitant reduction of mRNA expression, and concluded that the action of tamoxifen was based on interference in gene transcription[31].

This may be accounted partly by the presence of the GPR30 membrane receptor, whose activation is estrogen-mediated and which is independent of RE alpha and RE beta. However, the finding by Filardo and Thomas that RE antagonists can stimulate GPR30-dependent adenylcyclase is particularly important as regards antiestrogen therapy. Possibly, the most significant aspect is that antiestrogens, such as tamoxifen, acting on the GPR30 receptor, behave as true estrogen agonists able to trigger the release of growth factor (EGF) from breast cancer cells[32].

As shown in other studies from this group, there could be an extrinsic mechanism that does not initially involve the apoptotic mechanisms related to the BCL family, at least initially, since we found a significant increase of apoptotic corpuscles after 48 hours of treatment, but without changing BCL-2 positivity[17].

In brief, the small variation in positivity of the BAX and BCL-2 proteins after 14-day *in vivo* exposure to tamoxifen induces a reflection on the possibility that these proteins are not early altered in mammary carcinogenesis. It may also be that these proteins do not have any predictive values for response to endocrine therapy.

Future studies using molecular methods may put an end to doubts regarding the time of exposure to the drug. This is needed in order to interfere in the biomarkers and proteins from gene transcription related to programmed cell death.

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