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Expressão dos antígenos Ki-67, Bcl-2 e Bax no carcinoma de mama de mulheres tratadas com raloxifeno

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SUMÁRIO

SÍMBOLOS, SIGLAS E ABREVIATURAS

CRE: Complexo Receptor-Estrogênio

CoA: Coativadores

CORE: *Continuing Outcomes Relevant to Evista*

DNA: Ácido Desoxirribonucléico

E_2: Estrogênio

ERE: Elemento Responsivo Específico

FDA: *Food and Drug Administration*

INCA: Instituto Nacional do Câncer

MORE: *Multiple Outcomes of Raloxifene Evaluation*

NSABP P1: *National Surgical Adjuvant Breast and Bowel Project P-1*

RE: Receptor de Estrógeno

REα: Receptor de Estrógeno alfa

REβ: Receptor de Estrógeno beta

SERM: Modulador Seletivo dos Receptores de Estrógenos

STAR: *Study of Tamoxifen and Raloxifene*

TAF-1: Função de Ativação da Transcrição Gênica 1

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Introdução: A terapia endócrina é uma das mais importantes estratégias terapêuticas no combate ao câncer de mama. Entretanto, apesar da positividade dos receptores de estrógenos, nem sempre a resposta é favorável. Portanto, é grande o interesse dos pesquisadores em encontrar biomarcadores que possam avaliar o prognóstico, assim como predizer a resposta terapêutica. **Objetivo**: Avaliar o efeito do raloxifeno na expressão do Ki-67, Bcl-2 e Bax no carcinoma ductal invasivo receptor de estrógeno positivo. **Pacientes e Métodos:** Vinte mulheres pós-menopausadas, que fizeram uso de 60 mg de raloxifeno via oral diariamente por 28 dias, previamente a cirurgia definitiva, participaram deste estudo. Duas amostras de cada paciente foram obtidas por biópsia incisional, uma por ocasião da confirmação do diagnóstico de carcinoma ductal invasivo e avaliação do *status* dos receptores de estrógeno, e outra, 29 dias depois, por ocasião da cirurgia definitiva. Avaliação imunoistoquímica foi realizada nas amostras tumorais previamente e após tratamento com raloxifeno para avaliar a expressão do Ki-67, Bcl-2 e Bax. Os testes de Friedman e McNemar foram usados na análise dos dados e a significância estatística estabelecida em 5%. **Resultados:** A percentagem média de núcleos corados pelo Ki-67 foi de 24,86 ± 2,95 previamente ao tratamento com raloxifeno e 13,33 ± 1,52 após o tratamento ($p<0,001$). Antes do tratamento com raloxifeno, somente 9/20 casos (45%) foram classificados como Bcl-2 positivos, enquanto após o tratamento 17/20 (85%) foram classificados como Bcl-2-positivos ($p<0,013$). Com relação à expressão do Bax, 11/20 pacientes (55%) foram classificados como positivos para expressão do Bax previamente ao tratamento com raloxifeno, enquanto que 9/20 (45%) foram classificadas como positivos após tratamento com raloxifeno ($p=0.479$). **Conclusão:** O raloxifeno reduziu significantemente a expressão do antígeno Ki-67, aumentou a expressão do antígeno Bcl-2 e não alterou a expressão do Bax no carcinoma ductal infiltrante de mama de mulheres pós-menopausadas.

Palavras-chave: Câncer de mama, SERMs, Raloxifeno, Ki-67, Bcl-2, Bax.

ABSTRACT

Background: Breast cancer endocrinotherapy is one of the most important therapeutic strategies. Nevertheless, in spite of the positivity estrogen receptor, not always there is a good response. Therefore, there is a great interest of the researchers in finding biomarkers to evaluate the prognostic, and predict therapeutic responses. **Objective**: The aim of this study was to evaluate the effect of raloxifene on Ki-67, Bcl-2 and Bax expression in estrogen-receptor-positive invasive ductal breast carcinomas. **Patients and Methods:** Twenty postmenopausal women who had taken 60 mg of raloxifene daily for 28 days prior to definitive surgery were enrolled in the study. Two tumor samples were obtained by incisional biopsy during the study, one at the time of confirmation of the diagnosis of invasive ductal carcinoma and evaluation of estrogen receptor status, and the other 29 days later, at the time of definitive surgery. Immunohistochemistry was performed in tumor samples prior to and following raloxifene treatment to evaluate Ki-67, Bcl-2 and Bax expression. The Friedman and McNemar tests were used in the statistical analysis of the data, significance being established at 5%. **Results**: The mean percentage of Ki-67-stained nuclei was 24.86 ± 2.95 prior to raloxifene treatment and 13.33 ± 1.52 following treatment ($p<0.001$). Prior to raloxifene treatment, only 9/20 cases (45%) were classified as Bcl-2-positive, whereas following treatment 17/20 (85%) were classified as Bcl-2-positive ($p<0.013$). With respect to Bax expression, eleven of the 20 patients (55%) were classified as positive for Bax expression prior to raloxifene treatment, while 9(45%) were classified as positive following raloxifene treatment ($p=0.479$) **Conclusion:** The raloxifene treatment significantly reduced Ki-67 antigen expression, increased Bcl-2 expression and did not alter Bax expression in estrogen receptor-positive breast carcinomas of postmenopausal women.

Keywords: Breast cancer, SERMs, Raloxifene, Ki-67, Bcl-2. Bax.

1. INTRODUÇÃO

A terapia endócrina hoje constitui uma das principais armas na prevenção e tratamento do câncer de mama, sendo realizada por meio dos Moduladores Seletivos dos Receptores de Estrógenos (SERMs) (Lewis & Jordan, 2005). A chave para o desenvolvimento da terapia endócrina foi a descoberta dos receptores de estrógenos como mediadores da ação estrogênica. A identificação destes receptores forneceu um mecanismo para descrever a ação estrogênica nos órgãos alvos, um teste para estabelecer o prognóstico e predizer a resposta à terapia endócrina no câncer de mama e um alvo foi identificado para o desenvolvimento de novas drogas para o tratamento e prevenção do câncer de mama (Ariazi *et al*., 2006).

O tamoxifeno e o raloxifeno representam os principais SERMs estudados na terapia endócrina, com o tamoxifeno sendo utilizado tanto no tratamento adjuvante quanto na quimioprevenção do câncer de mama em mulheres com alto risco para esta neoplasia. Por sua vez, o raloxifeno foi aprovado recentemente na quimioprevenção do câncer de mama em mulheres de alto risco na pós-menopausa (Rastogi, 2008).

Contudo, nem sempre os tumores de mama respondem adequadamente a terapia endócrina, apesar da positividade dos receptores de estrógenos, o que tem motivado a busca por outros biomarcadores com valor prognóstico e avaliadores da resposta terapêutica (Tanaka, 2004; Lewis & Jordan, 2005).

Para o estudo de novos biomarcadores, pesquisas avaliando o efeito de drogas administradas por curtos períodos de tempo (2-4 semanas) têm sido usadas. Biomarcadores, como os relacionados à proliferação celular e apoptose, apresentam a vantagem de poderem sofrer alterações em seus níveis antes de qualquer resposta clinica ao tratamento, e assim poderiam selecionar pacientes que se beneficiariam do tratamento adjuvante (Dowsett *et al.,* 1999). No entanto, estudos desse tipo envolvendo o raloxifeno são escassos, pois a maioria dos estudos relacionados a este SERM são clínicos e epidemiológicos e avaliam o efeito do raloxifeno na quimioprevenção (Da Silva *et al.,* 2006).

A proliferação celular e apoptose são fenômenos que parecem ser influenciados pela ação estrogênica, contudo, até onde pesquisamos, poucos são os estudos avaliando a efeito do raloxifeno na proliferação celular, avaliada pelo Ki-67 (Dowsett *et al*., 2001; Da Silva *et al.,* 2009), e nenhum estudo avaliou o efeito deste SERM na expressão dos antígenos Bcl-2 e Bax. Efeitos benéficos do raloxifeno no perfil desses biomarcadores, quando utilizado por um curto período de tempo, como tratamento primário, poderiam predizer a resposta terapêutica à endocrinoterapia, fim para o qual este SERM ainda não foi aprovado, o que nos levou a concepção do presente estudo.

2. REVISÃO DA LITERATURA

O câncer é uma das principais causas de mortalidade e morbidade no mundo, com mais de dez milhões de casos novos e mais de seis milhões de mortes por ano. De todas as neoplasias, exceto o câncer de pele não-melanoma, o câncer de mama é o mais freqüente nas mulheres em todo o mundo, sendo estimado cerca de um milhão de casos novos e seiscentas mil mortes por ano pela doença (Trufelli *et al.*, 2008).

No Brasil, segundo a "Estimativa de Incidência de Câncer no Brasil para 2008", publicada pelo Instituto Nacional do Câncer (INCA), o câncer de mama é o segundo mais incidente, abaixo apenas do câncer de pele não-melanoma, com 49.400 casos novos, dentre estes, 7.630 casos na região Nordeste. No Piauí, foi estimada uma taxa bruta de incidência de 20,8 novos casos de câncer de mama por cem mil mulheres (INCA, 2008).

A influência dos hormônios ovarianos no desenvolvimento do câncer de mama é notória, sendo comprovada desde que a ooforectomia foi instituída como um dos primeiros tratamentos do câncer de mama metastático, no final do século XIX (Gadducci *et al.,* 2005; Swaby *et al*., 2007). O estrogênio promove a proliferação celular do epitélio mamário normal e neoplásico, atuando principalmente por meio dos receptores de estrógenos, que estão presentes em cerca de 60 a 70 por cento

dos carcinomas mamários (Sutherland *et al*., 1998; Russo *et al*., 2001; Jalava *et al*., 2005).

Os receptores de estrógenos (RE) são proteínas intranucleares, sendo descrito dois tipos: o receptor alfa (REα) e o receptor beta (REβ), cujos genes estão localizados nas regiões cromossômicas 6q25.1 e 14q23.2, respectivamente, guardando grande homologia entre si (Ariazi *et al.,* 2006). Apresentam diferente distribuição e proporção nos tecidos humanos, com o REα sendo predominantemente expresso nos tecidos reprodutivos, mama, útero e vagina, e o REβ no sistema nervoso central, sistema cardiovascular, sistema imune, trato gastrointestinal, rins, pulmões e ossos (Kuiper *et al.,* 1997; Hall *et al.,* 1999; Gustafsson, 2000; Jordan *et al.,* 2003).

Os receptores de estrógenos, do ponto de vista estrutural, estão divididos em seis domínios funcionais: A, B, C, D, E e F. O domínio A-B, situado na extremidade amino-terminal do receptor, é responsável pela função de ativação da transcrição gênica 1 (TAF-1). O domínio C corresponde ao altamente conservado domínio de ligação ao DNA, responsável pela ligação a seqüências específicas nos genes promotores conhecidas como elemento responsivo ao estrógeno (ERE). O domínio D apresenta flexibilidade em sua estrutura secundária, permitindo as alterações de conformação da molécula do receptor durante sua ativação e dimerização. O domínio E é responsável pela função de ativação da transcrição gênica 2 (TAF-2), a qual pode ocorrer de forma independente ou sinérgica do TAF 1, dependendo do gene promotor ou do tipo celular. Finalmente, o domínio F, presente na extremidade

carboxi-terminal, parece modular a ativação da transcrição gênica (Figura 1) (Jordan, 2003; Sporn *et al.,* 2004).

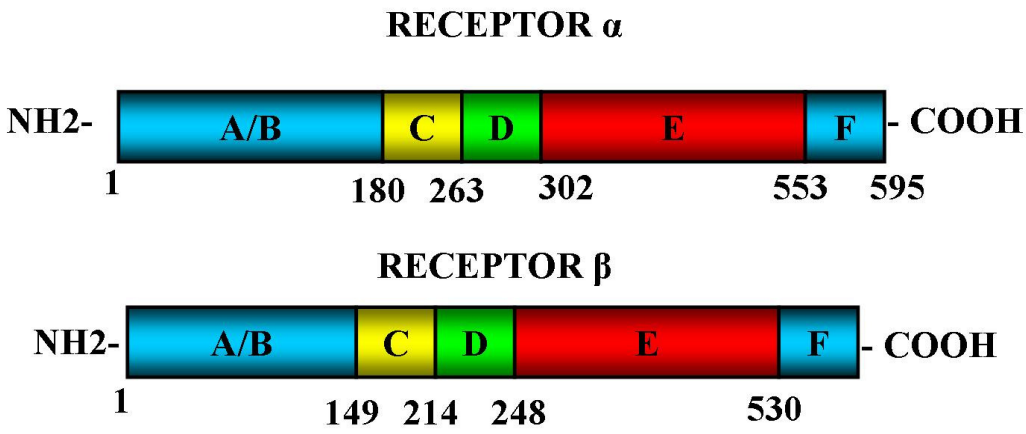

Figura 1: Representação esquemática dos receptores de estrógenos. Adaptado de Lewis *&* Jordan, 2005.

Os estrógenos, ao penetrarem no núcleo celular e se ligarem a seus receptores, promovem a ativação do complexo receptor-estrogênio (CRE) causando uma mudança na sua conformação, favorecendo a dissociação de proteínas e propiciando a dimerização do receptor. Subsequentemente, o CRE liga-se, por meio do domínio C, a pequenas seqüências palidrômicas do DNA chamadas de elemento responsivo específico (ERE), tipicamente localizadas na região promotora 5' dos genes responsivos aos estrogênios. Uma vez ligada ao DNA, são ativados os dois fatores de ativação transcricionais – TAF1 e TAF2 – que, após o recrutamento e ligação a coativadores e co-repressores que modulam a atividade transcricional dos genes responsivos ao estrogênio, resulta em resposta celular específica para o hormônio (Figura 2) (Jordan, 2003; Lewis & Jordan, 2005).

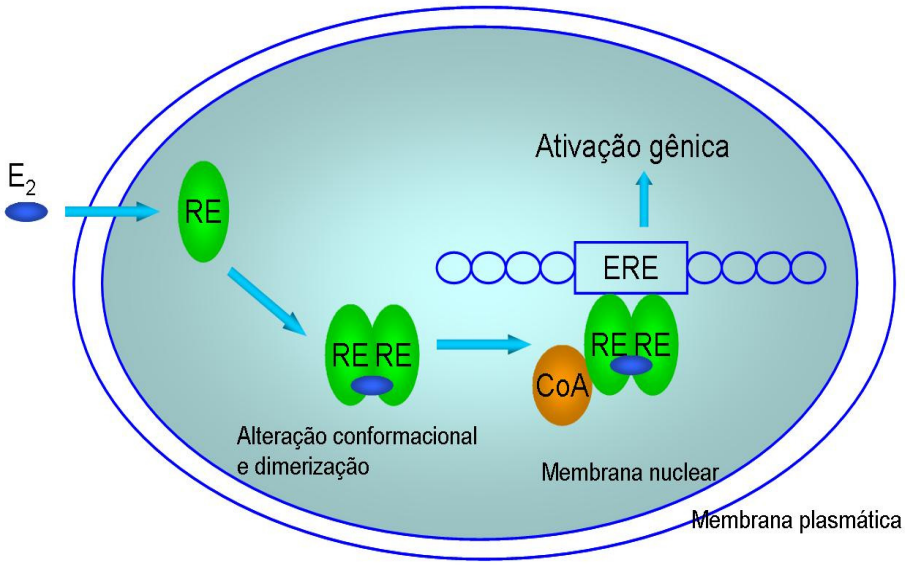

Figura 2: Representação esquemática do mecanismo de ação do estrogênio (E_2) por meio dos receptores de estrógenos (RE) nas células-alvo. O E_2 penetra na célula, ligando-se ao RE, induzindo sua mudança de conformação e dimerização. O complexo formado se liga aos elementos responsivos específicos (EREs) localizados na região promotora de genes responsivos e a transcrição gênica ocorre por ligação de coativadores moleculares (CoA). Adaptado de Lewis *&* Jordan, 2005.

O bloqueio desses receptores é uma das mais importantes estratégias na prevenção e tratamento do câncer de mama, sendo bastante estudada e empregada por meio do uso dos Moduladores Seletivos dos Receptores de Estrógenos (SERMs) (Saji & Kuroi, 2008; Cummings *et al.*, 2009). Os SERMs são drogas não-hormonais que agem principalmente por ligação aos RE em diversos órgãos, apesar de terem estrutura química diferente dos estrógenos (Cosman, 2003).

Os SERMs dividem-se em cinco grupos: os derivados trifeniletilenos (tamoxifeno, droloxifeno, idoxifeno, clomifeno, toremifeno), os derivados benzotiofenos (raloxifeno e arzoxifeno), sendo estas as duas principais classes químicas (Figura 3), além dos derivados tetrahidronaftalenos (lasofoxifeno,

trioxifeno, nafoxidino), derivados dos indóis (bazedoxifeno e pipendoxifeno) e dos derivados das benzopironas (ormeloxifeno, levormeloxifeno, EM-800, EM-652). Estas drogas atuam de forma agonista ou antagonista estrogênica, dependendo do tecido alvo e da espécie em que atuem (Diez-Perez, 2006, Da Silva *et al.,* 2005; Martino *et al*., 2005).

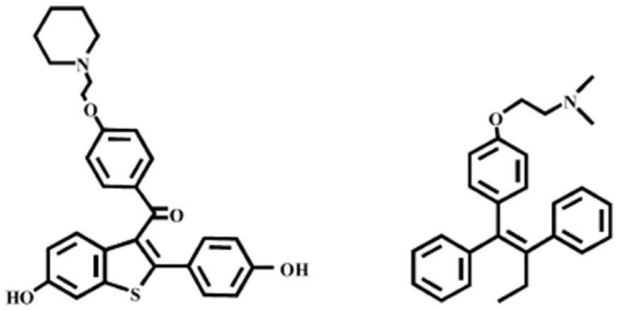

Figura 3: Representação esquemática das moléculas de raloxifeno e tamoxifeno. Adaptado de Lewis *&* Jordan*,* 2005.

O tamoxifeno é um SERM de primeira geração, aprovado em 1978 pelo *Food and Drug Admnistration* (FDA) dos Estados Unidos para o tratamento adjuvante de pacientes com câncer de mama e, em 1998, para a prevenção primária em pacientes de alto risco para câncer de mama, após mostrar uma redução neste grupo de pacientes de cerca de 49%, segundo os resultados do estudo *National Surgical Adjuvant Breast and Bowel Project P-1* (NSABP P-1) (Fisher *et al*., 1998, Rastogi, 2008). Contudo, quando utilizado por longos períodos, o tamoxifeno aumenta o risco do desenvolvimento de patologias endometriais, dentre elas o câncer de endométrio, em cerca de 2 a 7 vezes (Cuzick *et al.,* 2002; Singh *et al*., 2007). Isto tem motivado o estudo de outros SERMs, que apresentasse os mesmos

efeitos benéficos, com efeitos adversos menores que os do tamoxifeno, em especial sobre a estimulação endometrial.

O raloxifeno é um SERM de segunda geração, inicialmente utilizado no tratamento da osteoporose. Possui efeitos antiestrogênicos na mama, não acarretando efeitos proliferativos no endométrio (Cauley *et al*., 2001; Siris *et al*., 2005; Da Silva *et al*., 2006), além de promover outros efeitos positivos na mulher pós-menopausada, como alteração favorável do perfil lipídico e conseqüentes benefícios para o sistema cardiovascular (Delmas *et al.,* 1997; Muchmore *et al.,* 2000; Rastogi, 2008).

O real mecanismo de ação dos SERMs não está claro. Todavia, o tamoxifeno atua principalmente através dos seus metabólitos, o 4-hidroxi-tamoxifeno e o endoxifeno, que, ao se ligarem ao receptor de estrógeno, induzem uma mudança conformacional que bloquearia a TAF-2, predominante na mama, deixando livre a TAF-1, o que explicaria sua ação antagonista na mama e agonista no endométrio. Em contraste, a alteração conformacional induzida pelo raloxifeno no receptor de estrógeno bloquearia tanto a TAF-1 quanto a TAF-2, resultando em um efeito antiestrogênico tanto na mama quanto no endométrio (Black *et al*., 1983; Tzukerman *et al*., 1994; Marson *et al*., 1999).

O raloxifeno é administrado por via oral, sendo rapidamente absorvido no trato gastrointestinal, alcançando a concentração máxima plasmática de 12,5 ug/l em 0,5 horas. Após intenso fenômeno de primeira passagem pelo fígado, apresenta uma biodisponibilidade de apenas 2%, circulando com mais de 95 % de sua fração

ligada a proteínas plasmáticas. Apresenta meia vida de 28 horas, sendo excretado na bile e principalmente pelas fezes. Apenas 6% são eliminados pela urina como conjugados glicuronídeos (Balfour & Goa, 1998; Kemp *et al*., 2002). Tem como principais efeitos adversos câimbras nos membros inferiores, ondas de calor e tromboembolimo venoso, atingindo entre 1,8 a 2,6 mulheres em mil tratadas por ano (Goldstein *et al*., 2009).

Após a aprovação do tamoxifeno na quimioprevenção do câncer de mama, alguns estudos foram propostos para avaliar os efeitos do raloxifeno com o mesmo fim (Cummings *et al*., 1999; Cauley *et al.,* 2001; Siris *et al.,* 2005). Mais recentemente, o estudo STAR (*Study of Tamoxifen and Raloxifene*), que comparou o raloxifeno ao tamoxifeno na quimioprevenção do câncer de mama, mostrou uma redução no risco de câncer de mama invasivo promovido pelo raloxifeno similar a redução induzida pelo tamoxifeno, com menos eventos adversos como catarata e tromboembolismo (Vogel *et al.,* 2006). Os resultados deste estudo levaram a aprovação do raloxifeno na quimioprevenção do câncer de mama de mulheres na pós-menopausa pelo FDA em 2007 (Rastogi *et al.,* 2008).

Todavia, os estudos envolvendo o raloxifeno são clínicos e epidemiológicos e avaliam este SERM na quimioprevenção, enquanto que estudos experimentais sobre os efeitos diretos do raloxifeno no tratamento primário do câncer de mama são escassos (Da Silva *et al*., 2006). Apesar da terapia endócrina se constituir em uma das mais importantes formas de tratamento dos tumores de mama receptores de estrógeno positivo, nem sempre a resposta terapêutica é favorável, o que tem

despertado o interesse de pesquisadores em encontrar marcadores biológicos que possam avaliar não apenas o prognóstico, mais também predizer a resposta terapêutica (Tanaka, 2004).

Pesquisas avaliando o efeito de drogas, como SERMs e quimioterápicos, por curtos períodos de tempo no tratamento primário do câncer de mama têm sido realizadas com o objetivo de testar a resposta terapêutica de acordo com os parâmetros clínicos, tais como a redução do volume tumoral, quando administradas por 3 a 6 meses, ou alterações induzidas em biomarcadores quando administrados por um período de tempo mais curto (2 a 4 semanas) (Dowsett *et al.,* 2006). Os biomarcadores, como os de proliferação celular e apoptose, fenômenos biológicos essenciais ao crescimento tumoral, têm a vantagem de sofrerem alterações em seus níveis antes de qualquer resposta clinica ao tratamento, e assim poderiam selecionar pacientes que se beneficiariam do tratamento adjuvante (Dowsett *et al.,* 1999).

O equilíbrio entre a proliferação celular e a morte celular programada, conhecida como apoptose, é um determinante chave do crescimento e da progressão tumoral (Dowsett *et al.,* 1999). No câncer de mama, estes fenômenos são, portanto, de enorme importância na determinação da agressividade e do prognóstico dessa neoplasia (Schlotter *et al.,* 2008).

A avaliação rotineira da proliferação celular é feita na análise patológica do câncer de mama, sendo a quantificação da atividade mitótica o método tradicionalmente usado, entretanto, recentemente, a expressão imunoistoquímica do

Ki-67 tem sido largamente utilizado, por tratar-se de um método mais sensível de avaliação da atividade proliferativa (Elston & Ellis, 1991; Scott *et al.,* 1991; Fitzgibbons *et al*., 2000; Da Silva *et al.,* 2009). O Ki-67 é uma proteína nuclear, de função desconhecida, expressa em todas as fases do ciclo celular, exceto na fase G0 (fase de repouso) (Gerdes, 1990; Gerdes *et al.,* 1991; Fitzgibbons *et al*., 2000; Spyratos *et al.,* 2002). O papel da expressão imunoistoquímica do Ki-67 tem sido largamente investigado e a maioria dos estudos evidencia uma associação direta da elevação da expressão imunoistoquímica do Ki-67 com tumores receptores de estrógenos negativos, com maior grau nuclear e com maior atividade proliferativa, características relacionadas com um pior prognóstico (Tan *et al*., 2005).

Por sua vez, a apoptose, sendo um complexo mecanismo fisiológico de autodestruição, é responsável pela homeostase tecidual eliminando células danificadas e potencialmente prejudiciais (Schlotter *et al.,* 2008). A apoptose é regulada principalmente pelas proteínas da família bcl-2, que se dividem em duas classes opostas: uma classe de proteínas com atividade pró-apoptótica, representada pelo Bad, Bax e o Bak, e outra classe antiapoptótica, representada Bcl-2, Bcl-x, Bcl-w, Mcl-1 e A1/Bfl-1 (Sjöström *et al.,* 2002; Furtado-Veloso *et al*., 2008), destacando-se as proteínas Bcl-2 e o Bax, entre as mais estudadas. Além destas, participam também de forma essencial na apoptose, as mitocôndrias, o citocromo c e as caspases, entre outros (Reed, 2000).

A proteína Bcl-2 é considerada antiapoptótica e sua ação na gênese de tumores tem sido demonstrada em modelos animais e alguns tipos de tumores com os linfomas (Silvestrini *et al.,* 1994; Kroemer, 1997; Dos Santos *et al.,* 2008). É

codificada pelo proto-oncogene *bcl-2*, sendo primeiro descrito em linfomas de células B, como resultado da translocação que justapõe o gene *bcl-2*, no cromossomo 18 com o gene da imunoglobulina de cadeia pesada (IgH) no cromossomo 14, t(14;18), levando a uma superexpressão do Bcl-2, permitindo as células tornarem-se resistentes à apoptose (Leek *et al*., 1994).

Quanto à proteína Bax, trata-se de um membro pró-apóptótico da família bcl-2, que controla importantes etapas durante o processo apoptótico (Konstantinidou *et al.,* 2002). A perda da expressão da proteína Bax esta associada com um risco reduzido de recidiva ou uma maior sobrevida livre de doença em crianças com leucemia linfocítica aguda e discrasias de células plasmáticas (Renner *et al.,* 2000). Contudo, estudos em vários subgrupos de pacientes com câncer de mama diferem em seus resultados quanto ao impacto prognóstico da expressão da proteína Bax (Krajewski *et al.,* 1995; Veronesi *et al*., 1998; Daidone, 2000; Cannings *et al*., 2007).

Embora muitos caminhos possam existir, duas vias são atualmente propostas para regular a apoptose nas células mamárias: uma via extrínseca da qual participam os receptores de morte e um via intrínseca, do qual participa a mitocôndria (Figura 4). Na via extrínseca, os receptores de morte da família do Fator de Necrose Tumoral, presentes na superfície celular, após serem ativados por ligantes específicos, recrutam e se ligam a proteínas adaptadoras no citosol, promovendo a ativação de uma cadeia de proteínas conhecidas como caspases, iniciada, na via extrínseca, pela ativação da caspase-8, resultando na indução da apoptose (Reed, 1999, 2000; Otsuki, 2004).

Por sua vez, na via intrínseca ou mitocondrial, após um estímulo apoptótico, a mitocôndria libera o citocromo c que se liga a uma proteína de ativação das caspases conhecida como Apaf-1, desencadeando a ativação em seqüência das caspases, iniciada primeiro nesta via pela ativação da caspase-9, resultando na morte celular (Reed, 2000; Morishima *et al.,* 2008). As proteínas da família bcl-2 atuam nesta via, regulando a liberação do citocromo c pela mitocrôndria (Konstantinidou *et al.,* 2002). O Bcl-2, presente na superfície externa da membrana mitocondrial, inibe a apoptose, por meio da supressão da liberação do citocromo c pela mitocôndria, por conseguinte, impedindo o desencadeamento do sinal apoptótico, enquanto que o Bax induz ou facilita a liberação desta proteína ativadora das caspases, ainda que por mecanismos não totalmente elucidados (Reed, 1999, 2000; Ruvolo *et al.,* 2001).

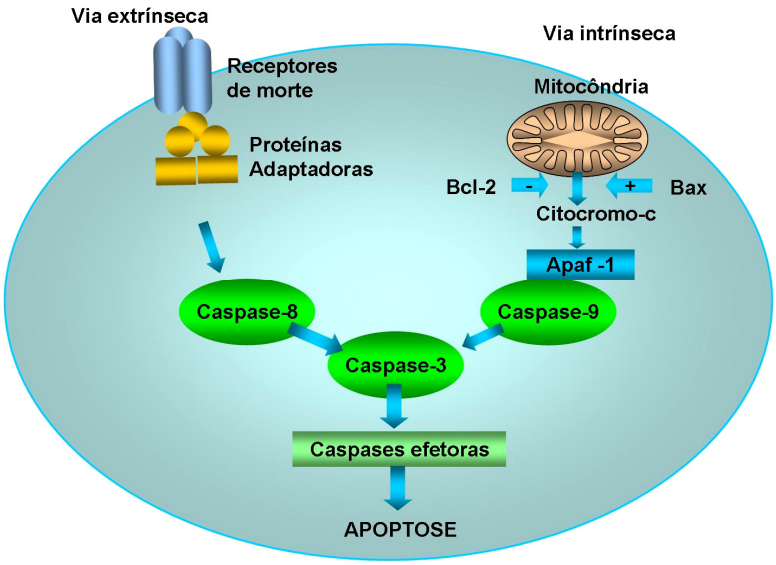

Figura 4: Representação esquemática das vias extrínseca e intrínseca da apoptose. Adaptado de Reed, 2000.

O potencial tumorigênico da proteína Bcl-2 tem sido demonstrado em modelos animais e alguns tipos de linfomas. Contudo, em muitos tumores humanos, incluindo o câncer de mama, o Bcl-2 paradoxalmente parece exercer um efeito supressivo tumoral (Pietenpol *et al.,* 1994; Borner, 1996). Uma meta-análise recentemente publicada mostrou que a expressão aumentada do Bcl-2 associa-se a características prognósticas favoráveis como o baixo grau nuclear e a positividade dos receptores de estrógenos, além de um maior tempo livre de doença e sobrevida global, independentemente do *status* linfonodal, tamanho do tumor e grau histológico (Callagy *et al,* 2008).

Alguns estudos têm avaliado o efeito de SERMs na proliferação celular e a expressão imunoistoquimica do Bcl-2 nos tumores mamários. Keen *et al.,* (1994) avaliaram o efeito de 20 mg/dia de tamoxifeno por 3 meses encontrando uma redução significativa da proliferação celular, avaliada pelo Ki-S1, enquanto que um tendência similar, mas não significativa, foi observada na expressão do Bcl-2, entre os tumores que reduziram de volume após o tratamento. Resultado este similar ao encontrado no estudo de Cameron *et al.,* (2000), que também avaliaram o efeito de 20 mg/ dia de tamoxifeno por três meses na proliferação celular, avaliado pelo Ki-S1, e na expressão do Bcl-2, encontrando uma redução significativa da expressão do Ki-S1 e uma tendência de redução na expressão do Bcl-2, embora não estatisticamente significante.

Contudo, em estudo realizado por Johnston *et al.,* (1994), observou-se uma redução significativa na proliferação celular, desta vez avaliada pelo Ki-67 e um aumento também significativo na expressão do Bcl-2 no carcinoma de mama

receptor de estrógeno positivo tratado com 20 mg de tamoxifeno por 18 dias a 3 meses. Os achados deste estudo sugerem que a redução na proliferação celular, induzida pelo tamoxifeno, estaria associada a um aumento na expressão do Bcl-2.

Há evidências *in vitro* que o gene *bcl-2* interage com o gene *c-myc* na regulação tanto da proliferação celular quanto da apoptose (Bissonnette *et al*., 1992, Fanidi *et al.,* 1992), O *c-myc* é um oncogene essencial na codificação do maquinário proliferativo das células, cuja expressão desregulada está implicada na gênese de muitas neoplasias (Evan *et al.,* 1994). É possível que a capacidade das células do câncer de mama passar para apoptose esteja retardada pelos profundos efeitos antiproliferativos da terapia antiestrogênica (Johnston *et al.,* 1994). É conhecido que o tamoxifeno reduz a expressão do *c-myc*, tanto em linhagens de células do câncer de mama RE positivo (Santos *et al.,* 1988) quanto no câncer de mama *in vivo* (Le Roy *et al*., 1991). Além do mais, pelo menos 20 proteínas da família bcl-2 tem sido descritas e o efeito de drogas, como os SERMs, no conjunto destas proteínas, assim como estas proteínas agem sinergicamente entre si determinando a apoptose, permanece incerto (Callagy *et al.,* 2008).

Todavia, estudos avaliando o efeito do raloxifeno na proliferação celular e na apoptose do câncer de mama são escassos. Um estudo realizado por Dowsett *et al.,* (2001) avaliando o efeito do raloxifeno, na dose de 60 e 600 mg por 14 dias, na expressão do Ki67 e na apoptose, por meio da quantificação dos corpos apoptóticos, mostrou uma redução significativa na proliferação celular, sem qualquer alteração na apoptose.

Recentemente, em um estudo realizado em nosso serviço, por Da Silva *et al.,* (2009), o raloxifeno reduziu significantemente a proliferação celular, avaliado pelo Ki-67, assim como a angiogênese, provavelmente pela redução na proliferação das células endoteliais, confirmada pela diminuição da expressão da proteína CD34, marcador de células endoteliais, no carcinoma ductal infiltrante de mama.

Por sua vez, também são poucos os estudos avaliando o efeito de SERMs, particulamente o raloxifeno, na expressão da proteína Bax em tumores de mama. Um estudo em linhagens de células do câncer de mama MCF-7 tratadas com tamoxifeno por 72 h não encontrou diferença significativa nos níveis de mRNA antes e após tratamento com este SERM (Zhang *et al.,* 1999). Este efeito foi diferente do encontrado por Strohmeier *et al.*(2002), que observou um aumento na expressão da proteína Bax em linhagens de células BT-20 do câncer de mama após 24 h de tratamento com tamoxifeno.

Veronesi *et al.* (1998) avaliaram o efeito do tamoxifeno no tratamento adjuvante de 58 pacientes com câncer invasivo de mama por um período prolongado de 5 anos e também não encontrou qualquer diferença na expressão da proteína Bax após tratamento. Contudo, estes autores encontraram uma associação entre tumores com expressão da proteína Bax aumentada e o envolvimento linfonodal axilar. Em outro estudo, avaliando o valor prognóstico da proteína Bax em 145 mulheres portadoras de câncer de mama, com mais de 70 anos de idade, submetidas a tratamento cirúrgico definitivo, seguido de tratamento adjuvante com tamoxifeno por 1 ano, não foi observado qualquer relação entre a expressão do Bax

e sobrevida livre de doença (Daidone, 2000). Estes achados são similares aos encontrados recentemente por Cannings *et al*. (2007).

Poucos estudos *in vitro* avaliaram a expressão da proteína Bax em linhagens de células tratadas com tamoxifeno (Salami *et al.,* 2003; Kim *et al.,* 2005). Contudo, nenhum estudo *in vitro* ou *in vivo* avaliou o efeito do raloxifeno na proteína Bax em células do câncer de mama. Um estudo usando a técnica de *Western blot* avaliou o efeito do raloxifeno na expressão da proteína Bax em linhagens de células do câncer de próstata e bexiga, não sendo observada qualquer alteração na expressão deste marcador (Kim *et al.,* 2002). Mais recentemente, no nosso meio, Furtado-Veloso *et al.* (2008) avaliaram o efeito de 60 mg de raloxifeno administrados por 22 dias na expressão da proteína Bax em tecido mamário normal adjacente ao fibroadenoma em mulheres no menacme, não encontrando qualquer alteração significativa na expressão desta proteína após o tratamento com este SERM.

Portanto, mesmo diante das controvérsias existentes, a proliferação celular e apoptose são fenômenos influenciados pela ação estrogênica (Johnston *et al.,* 1994; Schlotter *et al.,* 2008). Assim, a modulação da ação estrogênica nestes parâmetros, com o mínimo de efeitos colaterais, seria uma importante estratégia terapêutica no combate ao câncer de mama, além do que, os efeitos benéficos do raloxifeno no perfil dos biomarcadores Ki-67, Bcl-2 e Bax, quando utilizado por um curto período de tempo, como tratamento primário, poderiam predizer a resposta terapêutica à endocrinoterapia. Contudo, o raloxifeno ainda não foi testado e aprovado para este fim e estudos avaliando o efeito desta droga em biomarcadores de proliferação

celular e apoptose no câncer de mama são escassos na literatura, o que nos levou à concepção do presente estudo.

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4. OBJETIVOS

4.1. Objetivo Geral

Avaliar os efeitos do raloxifeno em biomarcadores no carcinoma ductal invasivo de mama receptor de estrógeno positivo de mulheres pós-menopausadas.

4.2. Objetivos específicos

Artigo 1

Avaliar o efeito do raloxifeno na expressão do Ki-67 e Bcl-2 no carcinoma ductal invasivo de mama receptor de estrógeno positivo de mulheres pós-menopausadas

Artigo 2

Avaliar o efeito do raloxifeno na expressão da proteína Bax no carcinoma ductal invasivo de mama receptor de estrógeno positivo de mulheres pós-menopausadas

5. PUBLICAÇÕES

Artigo 1:

Evaluation of Ki-67 and Bcl-2 antigen expression in breast carcinomas of women treated with raloxifene

Aceito para publicação em *Cell Proliferation* em 15 de junho de 2009.

Artigo 2:

The effect of raloxifene on Bax protein expression in breast carcinomas of postmenopausal women

Submetido para *Biomarkers* em 17 de Agosto de 2009.

5.1. Artigo 1

Evaluation of Ki-67 and Bcl-2 antigen expression in breast carcinomas of women treated with raloxifene

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Abstract

Objectives: to evaluate the effect of raloxifene on Ki-67 and Bcl-2 antigen expression in operable, stage II, estrogen-receptor-positive invasive ductal breast carcinomas. ***Materials and Methods:*** Twenty postmenopausal women who had taken 60 mg of raloxifene daily for 28 days prior to definitive surgery were enrolled in the study. Two tumor samples were obtained by incisional biopsy during the study, one at the time of confirmation of the diagnosis of invasive ductal carcinoma and evaluation of estrogen receptor status, and the other 29 days later, at the time of definitive surgery. Immunohistochemistry was performed in tumor samples prior to and following raloxifene treatment to evaluate Ki-67 and Bcl-2 expression. The Friedman and McNemar tests were used in the statistical analysis of the data, significance being established at 5%. ***Results****:* The mean percentage of Ki-67-stained nuclei was 24.86 ± 2.95 prior to raloxifene treatment and 13.33 ± 1.52 following treatment ($p<0.001$). Prior to raloxifene treatment, only 9/20 cases (45%) were classified as Bcl-2-positive, whereas following treatment 17/20 (85%) were classified as Bcl-2-positive ($p<0.013$). ***Conclusions:*** The raloxifene treatment significantly reduced Ki-67 antigen expression and increased Bcl-2 expression in breast carcinomas of postmenopausal women.

Keywords: breast cancer; Ki-67; Bcl-2; raloxifene; proliferation; apoptosis.

Introduction

Selective estrogen receptor modulators (SERMs) are considered important tools in combatting estrogen-dependent breast cancer (1,2). Tamoxifen was the first SERM clinically approved for the chemoprevention and treatment of breast cancer; however, when used over long periods of time, tamoxifen may result in a 2-7-fold increase in the patient's risk of developing endometrial cancer (3). Recent clinical trials have shown raloxifene, a second-generation SERM approved for the prevention and treatment of osteoporosis (1,2,4-6), to be as effective as tamoxifen in reducing invasive breast carcinoma, while reducing the incidence of endometrial carcinoma (4,6). Nevertheless, few studies have evaluated raloxifene for the primary treatment of breast cancer (7,8).

Drugs have been used over short periods of time for the primary treatment of breast cancer in order to test therapeutic response according to parameters of clinical response such as tumor volume, when administered for 3 to 6 months, or alterations induced in biomolecular markers, such as those linked to the cell proliferation and apoptosis, when administered for shorter period of time (2 to 4 weeks), with the advantage of suffering alterations before any clinical response of the tumor to the treatment (9). Bcl-2 is a protein that belongs to a family of apoptosis-related proteins codified by a protooncogene, Bcl-2, and considered antiapoptotic. However, various studies have found that in breast cancer, Bcl-2 expression is paradoxically associated with a better prognosis (10-11).

Some studies have shown the antiproliferative and proapoptotic effects of tamoxifen in breast cancer (12-14); however, studies involving raloxifene are few (7,8). A study evaluating the effect of raloxifene on Ki-67 expression and apoptosis by quantifying apoptotic bodies showed a reduction in cell proliferation and no alteration in apoptosis (7). Nevertheless, despite of studies evaluating the effects of raloxifene on Ki-67 protein expression of breast tumors (7,8), to the best of our knowledge, no study has yet been carried out to evaluate the Bcl-2 expression in invasive, estrogen-receptor-positive breast carcinomas of postmenopausal women following primary treatment with raloxifene, which led us to design the present study.

Material 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 prior to study initiation. Twenty women receiving medical care at the Mastology Division of the *Getúlio Vargas* Hospital of the Federal University of Piauí, who had been menopausal for at least one year and who had an invasive ductal, estrogen-receptor-positive Her2-negative, operable, stage II (≥3 cm) breast carcinoma and no previous history of treatment for breast cancer or hormonal replacement therapy (HRT), were included in the study. Tumor size varied from 3 to 5 cm (mean 3.8 cm) and the mean age of patients was 60.5 years (range 49-72 years). The majority of patients were multiparas (90%) (Table 1).

Study design

The patients received 60 mg of raloxifene/day for a period of 28 days prior to definitive surgery, beginning immediately after receiving the results of a diagnostic incisional biopsy. Two tumor samples were obtained by incisional biopsy during the study, one at the time of confirmation of the diagnosis of invasive ductal carcinoma and evaluation of estrogen receptor status, and the other 29 days later, at the time of definitive surgery. Tumors with nuclear staining for estrogen receptor (ER) measured semiquantitatively as high (i.e. >10% immunoreactive cells) were considered positive.

Immunohistochemistry for Ki-67 and Bcl-2

For immunohistochemical evaluation of Ki-67 and Bcl-2 expression, the tumor samples were fixed in buffered formalin for a period of 12-24 hours and then cut into 3-µm-thick sections. Next, the samples were processed and stained with hematoxylin-eosin for confirmation of the diagnosis of ductal invasive carcinoma, following which the sections were deparaffinized in xylol for 5 minutes, dehydrated in absolute ethanol and washed in buffered saline solution at pH 7.4 for 5 minutes. Next, the sections were treated with 3% hydrogen peroxide (H_20_2), diluted in buffered solution, for 5 minutes to block the endogenous peroxide. To recover the antigen, the slides were placed in racks containing 0.21% citric acid (pH 6.0) and heated in a microwave oven for 15 minutes at maximum power. Phosphate-buffered saline containing Tween (PBS-Tween) was added to the slides after they had been cooled for 20 minutes. The tissue samples were incubated with primary mouse anti-Ki-67 monoclonal antibody (clone MIB1, Ref. M7240, Dako, Carpinteria, USA / 1:4800) and

mouse anti-Bcl-2 monoclonal antibody (clone 124, Ref. M0887, Dako, Carpinteria, USA / 1:2000) and incubated overnight at 4-8°C. Next, the slides were washed with PBS-Tween and instilled with secondary reagent (Anti-mouse BA 2000, Vector, Burlingame, USA), incubated for 60 minutes at room temperature, washed again with PBS-Tween and instilled with the ABC Elite detection system (PK 6100, Vector, Burlingame, USA), incubated for 45 minutes at room temperature, washed once again with PBS-Tween, instilled with DAB (Diaminobenzidine tetra-hydrochloride, Ref. D5637, Sigma, St. Louis, USA) and incubated for 5 minutes. Finally, the slides were washed with distilled water, counterstained with hematoxylin, stained with ammoniacal solution, dehydrated with absolute ethanol, passed through xylol series and mounted in Permount resin. The cells that expressed the Ki-67 and Bcl-2 proteins were identified by dark brown staining 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 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 color video-camera (Samsung digital camera CHC-370N, 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 Ltda. (São Paulo, Brazil) for image analysis. For Ki-67 expression, 1,000 cells were counted in each slide, whether stained by the anti-Ki-67 antibody or not, using a magnification of 400X, beginning with the area of greatest Ki-67 expression. In each

case, the percentage of stained cells was obtained from the ratio of the number of cells with stained nuclei and the total number of cells multiplied by 100. Likewise, Bcl-2 immunoreaction was evaluated semiquantitatively according to the criteria established by van Slooten et al. (15) taking the following parameters into consideration: intensity of cell coloration (I) and the fraction of stained neoplastic cells (F). The intensity of cell staining was classified as: 0 (negative), 1 (weakly stained), 2 (moderately stained) or 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 ≥ 3 were classified as positive for Bcl-2. In all cases, brownish staining in the cytoplasm was adopted as the standard for positivity (15).

Statistical Analysis

The Friedman nonparametric test was used to analyze the percentages of nuclei stained with anti-Ki-67 prior to and following raloxifene treatment (16). McNemar's test of symmetry was used to evaluate agreement between the classification of cells stained positive or negative with anti-Bcl-2 prior to and following raloxifene treatment (17). Significance was established at $p<0.05$.

Results

Under light microscopy, there was a greater concentration of Ki-67-stained nuclei in the samples obtained prior to raloxifene treatment compared to those collected following therapy. The percentage of cells intensely stained for Bcl-2 was higher in

the samples obtained following raloxifene treatment compared to the pretreatment samples (Figure 1). The mean percentage of Ki-67-stained nuclei was 24.86 ± 2.95 and 13.33 ± 1.52 ($p<0.001$) prior to and following raloxifene treatment, respectively (Figure 2). With respect to Bcl-2 antigen expression, of the 11 cases classified as negative for Bcl-2 expression prior to treatment, 8 were classified as positive following raloxifene use and only 3 remained negative, whereas all 9 cases that were classified as positive prior to treatment remained positive for Bcl-2 expression following treatment. Therefore, only 9 of the 20 patients (45%) were classified as positive prior to treatment, whereas following raloxifene treatment Bcl-2 expression was positive in 17 of the 20 samples (85%) ($p<0.013$) (Table 2).

Discussion

In the present study, raloxifene, administered at the dose of 60 mg/day for 28 days to postmenopausal women with ER-positive Her2-negative invasive ductal breast carcinoma, significantly reduced Ki-67 expression while significantly increasing Bcl-2 expression. The 60 mg dose of raloxifene was selected because this is the dose generally used for the prevention and treatment of osteoporosis and was also the dose used in the studies in which raloxifene was evaluated for the chemoprevention of breast cancer (4-6) . The 28-day schedule of use of the medication was chosen since 28 days is the normal delay incurred by a patient between her first consultation at this institute and surgical treatment; hence, the proposed treatment would result in no further delay in carrying out definitive surgery. Studies investigating the effect of drugs on breast cancer have generally used core biopsy to obtain tumor samples; however, taking the heterogeneity of the tumors into consideration, the small tumor

volume obtained with this type of biopsy implies that the fragment removed may not be representative of the tumor as a whole (18) and for this reason we opted to perform incisional biopsies.

To the best of our knowledge, this is the first study in which the effect of raloxifene was evaluated on the immunohistochemical expression of Bcl-2 in ER-positive Her2-negative invasive breast cancer samples from postmenopausal women. A study carried out by Dowsett et al. (7) used Ki-67 expression to evaluate the effect of 60 mg of raloxifene taken for 14 days on the proliferation of breast cancer and also reported a significant reduction in the expression of this marker. These investigators used the immunohistochemical technique of terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL), to calculate the apoptotic index and reported no significant difference prior to and following raloxifene treatment. These results differ from the findings of the present study in which raloxifene used for 28 days increased expression of the antiapoptotic protein Bcl-2. However, direct assessment of apoptosis is a more consistent method, while the behaviour of Bcl-2 may reflect other biological process within the transformed cell (7). Likewise, previous studies have shown that reduced proliferation is associated with Bcl-2 expression (13).

The antiproliferative effects of SERMs on breast cancer have been well documented in various studies showing their effect in reducing cell proliferation evaluated by Ki-67 immunohistochemical expression (8,12,13,19), as confirmed in the present study. The role of the immunohistochemical expression of Ki-67 has been widely investigated, the majority of studies showing a direct association between elevated

Ki-67 immunostaining and negative estrogen receptor immunohistochemistry, higher grade and highly proliferative tumors, characteristics known to be associated with poorer prognosis (20), although it has still not been possible to establish an appropriate and universally accepted cutoff limit to distinguish between high and low proliferative activity (21). Some authors use Ki-67 staining of 20% or more to define a high level, while others advocate segregation high from low staining via the median of positively stained cells as the distinguishing value (20,22,23). Additional variations issues include selection of the appropriate area for evaluation of immunostaining be it the center or periphery of the tumor; area of highest cell density; or zone of highest tumor cell immunohistochemical reactivity (10,20). In this study, percent positively stained nuclei and area of highest cell density were the variables used in the evaluation of Ki-67 expression pre- and post-treatment with raloxifene.

In the present study, the percentage of positivity for Bcl-2 expression was 45% prior to raloxifene use, which is close to the values found in other studies such as those carried out by Silvestrini et al. (24) and Johnston et al. (13), who reported, respectively, around 43% and 32% of positivity for the immunohistochemical expression of Bcl-2 in invasive breast tumors. Following raloxifene use, the percentage of positivity for Bcl-2 expression practically doubled, increasing from 45% to 85%, an increase proportionally similar to that found by Johnston et al. (13) who reported an increase in the percentage of Bcl-2-positive tumors from 32% to 65% following use of tamoxifen for a period of 18 days. Since we have been unable to find any previous studies evaluating Bcl-2 expression after raloxifene treatment, a direct comparison between the findings of the present study and previous findings is impossible.

Bcl-2 protein is considered antiapoptotic and its tumorigenic potential has been demonstrated in animal models and in some types of tumor such as lymphomas (25). Nevertheless, in many human tumors including breast cancer, Bcl-2 paradoxically appears to exert a suppressive effect on tumors and its expression is, instead, associated with favorable prognostic characteristics such as low nuclear grade and estrogen-receptor positivity (11,25). A metaanalysis of 25 studies involving 5,892 cases of breast cancer in which the prognostic role of Bcl-2 immunohistochemical expression was investigated showed that it is strongly associated with longer disease-free survival and overall survival, its effect being dependent neither on lymph node status, tumor size nor histological grade (11).

The mechanism by which Bcl-2 may exert its protective effect on breast cancer remains to be fully clarified. In vitro studies have shown that Bcl-2 exerts an inhibitory effect on the cell cycle, prolonging the G0 phase and therefore delaying the passage of the cell to the G1 phase (26). It is also possible that the capacity of breast cancer cells to proceed to apoptosis may be delayed by the profound antiproliferative effects of antiestrogenic therapy. It has also been reported that c-myc, a protooncogene that is essential in codifying the proliferative machinery and whose unregulated expression has been associated with many neoplasias, is a determinant of both cell proliferation and apoptosis and its expression is accentuated by estrogen and suppressed by antiestrogens (27). Furthermore, although at least 20 proteins of the Bcl-2 family have been described, the effect of drugs such as raloxifene on these proteins as a whole, as well as how they synergically affect apoptosis, remains unknown.

In conclusion, there was a significant reduction in proliferative activity as evaluated by Ki-67 immunohistochemical expression and a significant increase in Bcl-2 expression in estrogen-receptor-positive breast carcinoma of postmenopausal women treated with raloxifene. Our results suggest that raloxifene used for short period of time, before definitive surgery, may select that patients who would probably benefit from adjuvant endocrinotherapy with this drug; however, further basic and clinical studies must be carried out to clarify the biomolecular mechanisms of tumor response to raloxifene.

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Conflicts of interest: None.

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Table 1. Characteristics of patients

Characteristics	n	%
Age (years)		
40-49	2	10
50-59	6	30
60-69	10	50
≥ 70	2	10
Parity		
Nullipara	2	10
Multipara	18	90
Size of tumor (cm)		
3.0 – 3.9	14	70
4.0 – 5.0	6	30
Histological grade		
1	9	45
2	8	40
3	3	15
Staging		
IIa	10	50
IIb	10	50
Her2/neu positive	0	0

Table 2: Percentage of breast cancer cases with Bcl-2-positive cells prior to and following raloxifene treatment.

	Post-Treatment		
Pre-Treatment	Positive	Negative	Total
Positive	9	0	9 (45%)
Negative	8	3	11 (55%)
Total	17 (85%) *	3 (15%)	20 (100%)

* There was a statistically significant increase in Bcl-2 expression following raloxifene treatment ($p<0.013$).

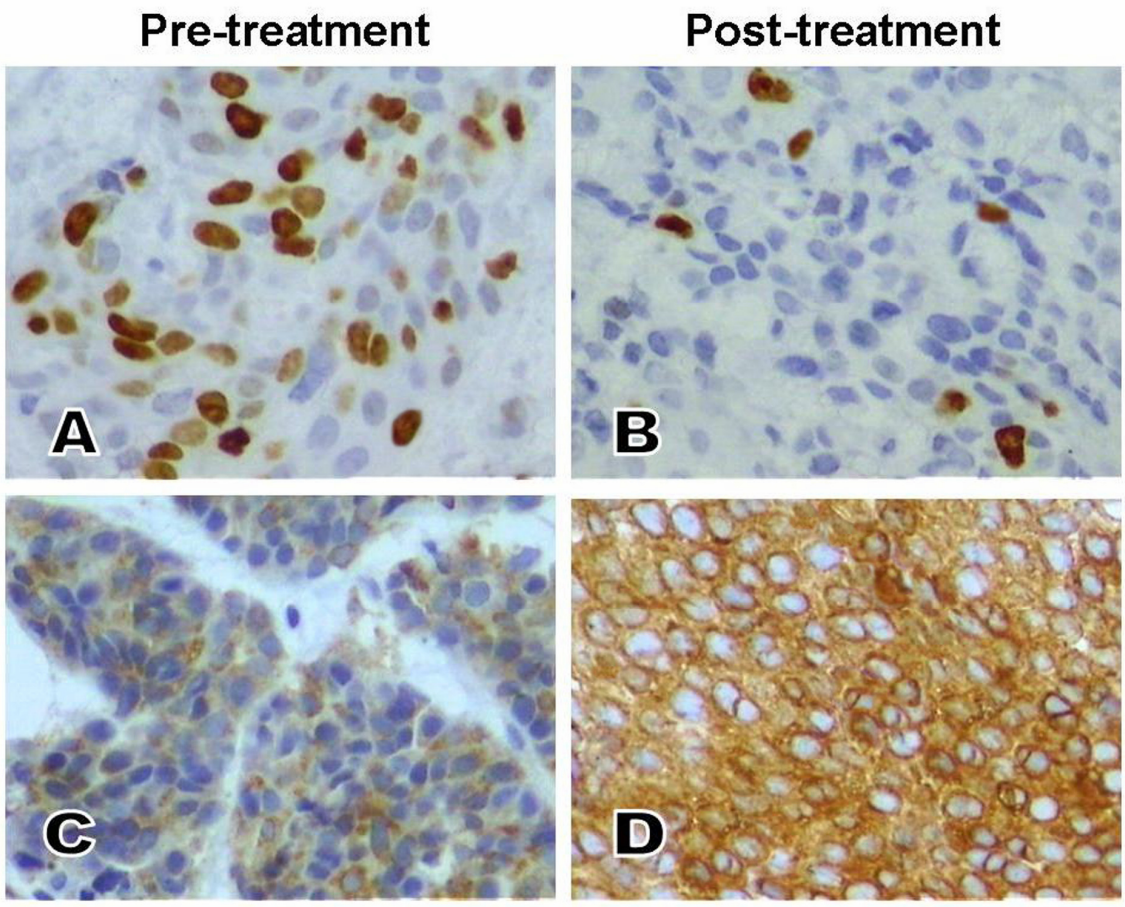

Figure 1. Photomicrographies of histological sections of breast cancer from patient # 4. Note numerous nuclei stained brown by the anti-Ki-67 antibody prior to treatment with raloxifene (a), and sparse stained nuclei post-treatment (b). Observe a negative immunohistochemical reaction for the Bcl-2 protein prior to treatment with raloxifene, as expressed by sparse cells with cytoplasms weakly stained in brown (c), and a positive immunohistochemical reaction post-treatment, as expressed by numerous cells with cytoplasms intensely stained (d) (original magnification 400X).

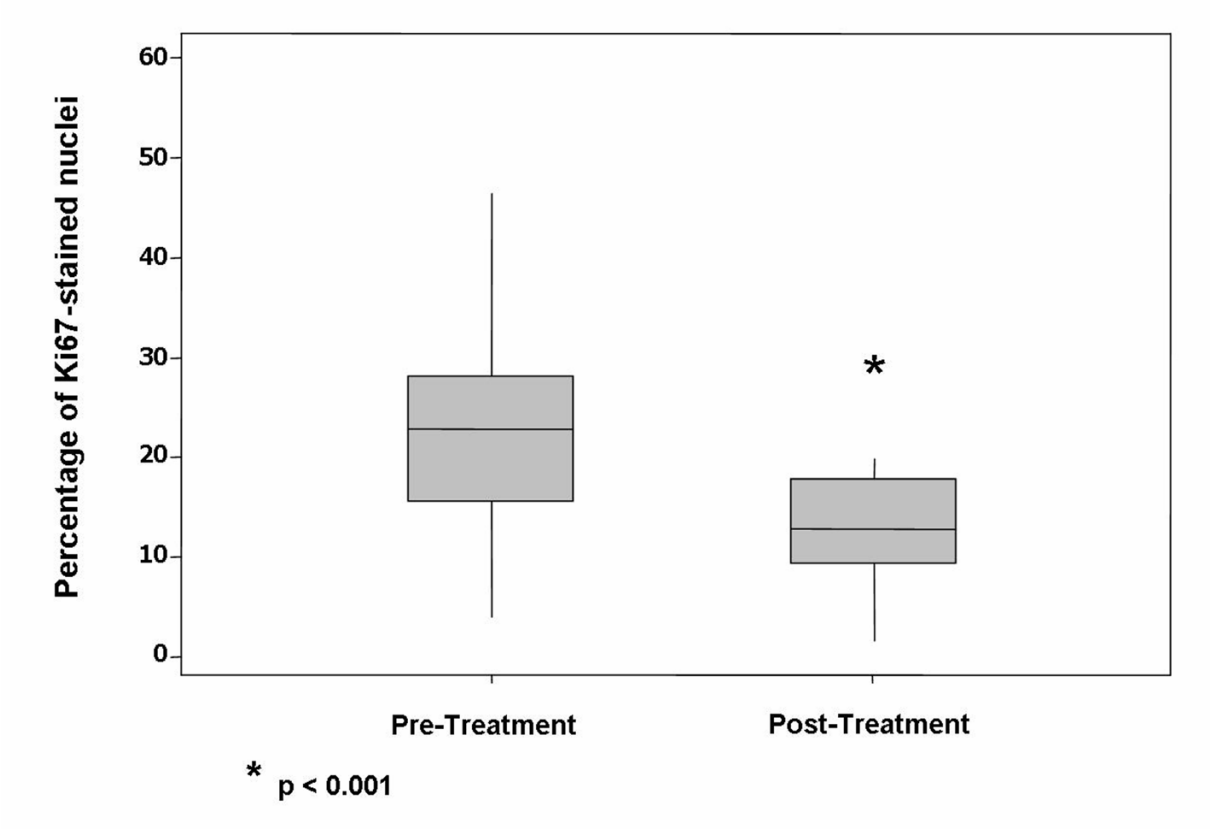

Figure 2. Boxplot of the mean percentage of nuclei stained with the anti-Ki-67

5.2. Artigo 2

The effect of raloxifene on Bax protein expression in breast carcinomas of postmenopausal women

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Running title: Raloxifene and Bax protein in breast carcinoma

Abstract

The aim of this study was to evaluate the effect of raloxifene on Bax protein expression in breast carcinomas of postmenopausal women. Twenty postmenopausal patients with operable stage II, estrogen receptor-positive, infiltrating ductal breast carcinoma were treated with oral raloxifene at a dose of 60 mg/day for a period of 28 days before definitive surgery. Tumor samples were obtained by incisional biopsy at the time of diagnosis and again at the time of definitive surgical treatment. Immunohistochemical evaluation of Bax expression was assessed semiquantitatively based on the fraction of stained tumor cells and intensity of staining. McNemar's test was used to analyze data ($p<0.05$). Eleven of the 20 patients (55%) were classified as positive for Bax expression prior to raloxifene treatment, while 9(45%) were classified as positive following raloxifene treatment ($p=0.479$). Raloxifene did not alter Bax expression significantly in estrogen receptor-positive breast carcinomas of postmenopausal women.

Keywords: apoptosis; breast; cancer; raloxifene; Bax; SERMs

Introduction

Raloxifene, a second-generation selective estrogen receptor modulator (SERM), first used in the treatment of osteoporosis, was recently approved by the Food and Drug Administration (FDA) for estrogen-positive breast cancer chemoprevention in high-risk postmenopausal women, following the results of a large clinical trial, named Study of Tamoxifen and Raloxifene (known as STAR) (Vogel et al. 2006, Rastogi, 2008). In the STAR trial, raloxifene was shown to reduce the risk of developing estrogen receptor-positive invasive carcinoma of the breast similar to tamoxifen, whereas it reduced the risk of endometrial carcinoma in contrast to tamoxifen (Swaby et al. 2007).

Nevertheless, studies evaluating the direct effect of raloxifene on biomarkers, such as those related to cell proliferation and apoptosis, both in normal and cancerous human breast tissue are scarce (Dowsett 2001, Da Silva 2006, 2009a, 2009b). Apoptosis, also known as programmed cell death, is an important mechanism of tissue homeostasis, removing damaged and potentially prejudicial cells (Reed 1999, Millen et al. 2006). It is regulated by the bcl-2 family of proteins. The bcl-2 family is divided into anti-apoptotic proteins such as Bcl-2, Bcl-xl and Bcl-w, and proapoptotic proteins such as Bad, Bid and Bax (Kumar et al. 2000, Reed 2000).

Bax is a protein that resides in the cell cytoplasm in an inactive state and participates in the mitochondrial or intrinsic pathway of apoptotic activation (Kim et al. 2002). Once activated, Bax forms oligomers in intracellular membranes, including the outer membrane of the mitochondria. Bax expression induces increased permeability,

leading to the release of proapoptotic proteins, such as cytochrome-c, which determines the activation of caspases, culminating in cell death (Cannings et al., 2007). Contrary to Bcl-2, whose prognostic value has already been demonstrated in several studies (Callagy et al. 2008, Dos Santos et al. 2008), the actual role of Bax protein as a prognostic biomarker that predicts response to therapy remains unclear (Veronese et al. 1998, Daidone et al. 2000, Cannings et al. 2007). Nevertheless, some authors have demonstrated that Bax may be involved in a mechanism of chemotherapy and SERM resistance in breast cancer (Krajewski et al. 1995).

Some previous studies examining the effects of tamoxifen on Bax expression in breast carcinoma have shown discordant results (Salami et al. 2003, Kim et al. 2005). However, up to now our investigation has not found any study evaluating the effect of raloxifene on Bax protein expression in estrogen receptor-positive invasive ductal carcinoma, which led us to conceive the present study.

Methods

Patients

Twenty postmenopausal patients with stage II operable (larger than 3 cm), infiltrating, estrogen receptor-positive, ductal carcinoma of the breast participated in this study. These women had received medical care at the Division of Mastology in the Department of Gynecology at Getúlio Vargas Hospital, *Universidade Federal do Piauí*. The study was approved by the Human Research Ethics Committee of the *Universidade Federal do Piauí*, in compliance with the Declaration of Helsinki of

1975, as revised in 1985. Informed consent was obtained from all participants before the study began. All patients had been menopausal for at least one year and had no previous history of breast cancer treatment. Tumor size varied from 3 to 5 cm (mean: 3.8 cm) and the mean age of the patients was 60.5 years (range: 49-72 years). All participants had negative HER-2 expression. The patients received oral raloxifene (60 mg/day) for 28 consecutive days prior to definitive surgery. Raloxifene therapy was initiated immediately after the patient received the result of the diagnostic incisional biopsy. Two tumor samples were obtained by incisional biopsy during the study. One sample was obtained when the diagnosis of infiltrating ductal carcinoma was confirmed and estrogen receptor status was evaluated. The other sample was obtained 29 days later, at the time of definitive surgery. The material was fixed in 10% buffered formalin for 12-24 hours, and then embedded in paraffin blocks. Tumors were considered positive, when semiquantitative evaluation of estrogen receptors was classified as high (≥10% immunoreactive cells) following immunohistochemical staining (Thike et al 2001).

Immunohistochemistry for Bax

For immunohistochemical evaluation of Bax expression, tumor samples were fixed in buffered formalin for a period of 12-24 hours and then cut into 3-µm-thick sections. The samples were then processed and stained with hematoxylin-eosin for diagnostic confirmation of ductal invasive carcinoma. Sections were deparaffinized in xylol for 5 minutes, dehydrated in absolute ethanol and washed in buffered saline solution at pH 7.4 for 5 minutes. Next, the sections were treated with 3% hydrogen peroxide (H_20_2), diluted in buffered solution for 5 minutes to block endogenous peroxidase activity.

For antigen retrieval, the slides were placed on racks containing 0.21% citric acid (pH 6.0), Phosphate-buffered saline containing Tween (PBS-Tween) was added to the slides after they had cooled for 20 minutes. The tissue samples were incubated overnight at 4-8°C with anti-Bax monoclonal antibody (clone: BGA, Ref. 3533, lot: 0057J, Dako, Carpinteria, USA/1:400 dilution). Next, the slides were instilled with Envision anti-rabbit (Dako K4003, Carpinteria, USA), incubated for 45 minutes at room temperature, washed once again with PBS-Tween, instilled with DAB (Diaminobenzidine tetra-hydrochloride, Ref. D5637, Sigma, St. Louis, USA) and incubated for 5 minutes. Finally, the slides were washed with distilled water, counterstained with hematoxylin, stained with ammoniacal solution, dehydrated with absolute ethanol, passed through xylol series and mounted on Permount resin. The cells that expressed Bax protein were identified by dark brown staining of the cytoplasm.

Quantification

Bax expression was evaluated under light microscopy by two observers, who were blinded to group identification. These observers semiquantitatively counted the cells with positively stained cytoplasm (under a magnification of 400x) using a system consisting of a light microscope (Nikon Eclipse E-400, optical microscope, Tokyo, Japan) connected to a videocamera (Samsung Digital Camera SCC-131, Seoul, Korea). The image was captured and transmitted to a computer equipped with the Imagelab® software program (Softium Informatica LTDA, São Paulo, Brazil). Immunoreaction was assessed according to criteria established by Van Slooten *et al.* (1996), taking the following parameters into consideration: the intensity of cell

staining (I) and the fraction of stained neoplastic 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 a combination of both parameters (I and F), ranging from 0 to 6. Cases with final scores ≥ 3 were classified as positive for Bax. In all cases, brownish staining of the cytoplasm was adopted as the standard for positivity. Assessment was initiated at the site of the largest quantity of stained cells; thereafter, other microscopic fields were randomly selected. The intensity of staining and percentage of stained tumor cells were assessed, resulting in the final score (Van Slooten et al. 1996).

Statistical analysis

McNemar's test of symmetry (Agresti 2002) was used to evaluate the level of agreement between positive and negative classifications of Bax before and after treatment with raloxifene in breast cancer patients. Statistical significance level was established at $p<0.05$.

Results

Under light microscopy, the concentration of cells stained brown by anti-Bax antibody in tumor samples obtained before raloxifene treatment was similar to the concentration of cells positive for Bax expression in the samples collected after raloxifene treatment (Figure 1). Quantitative analysis of 20 cases classified according to Bax expression showed that 9 patients classified as negative before

treatment, remained negative after treatment. Of the 11 patients who were classified as positive before treatment, 2 became negative after treatment, while 9 remained positive. Therefore, 55% of the patients (11/20) were classified as positive for Bax before raloxifene treatment, while 45% (9/20) were classified as positive after raloxifene treatment which was not a statistically significant difference (p=0.479) (Table I).

Discussion

In this study, oral raloxifene at a dose of 60 mg/day, which is a dose normally used for the treatment of osteoporosis and breast cancer chemoprevention, did not induce any alteration in Bax immunohistochemical expression when administered for 28 days. The duration of treatment was chosen by a current preference to evaluate drug effect on biomarkers for short time periods (2-4 weeks). This is a more inexpensive and practical intervention, causing no delay in definitive surgical treatment. It may also provide important information on prognostic factors and tumor response to treatment (Dowsett et al. 2006). Tumor samples were obtained by incisional biopsies at the moment of breast cancer diagnosis and during the definitive surgery. In this type of biopsy, larger tissue fragments are removed. The risk of obtaining areas that are not representative of the whole tumor is reduced, as may occur with core biopsy (Jacobs et al. 1998).

Some studies have report pro-apoptotic effects of SERMs (Chen et al. 1996, Cameron et al. 2000) reason why Bax protein was chosen for this study, since it is the principal pro-apoptotic marker among the proteins of the bcl-2 family (Reed,

1999, 2000). In this study, the positive rate of Bax protein expression in tumors before treatment was 55%, which was very close to that reported by other studies involving nontreated tumors (Krajewski et al. 1995, Kapucuoglu et al. 1997, Veronese et al. 1998, Daidone et al. 2000). Tumors were positive for Bax in 45% of the patients after treatment with raloxifene and this was not a statistically significant difference. Studies evaluating the effect of SERMS on Bax expression have shown conflicting results and overmore, studies evaluating the effect of raloxifene in breast tumors are scarce. A study of MCF-7 breast cancer cell lines treated with tamoxifen for 72 h found no significant difference in mRNA levels before and after treatment with this type of SERM (Zhang et al. 1999). This effect was different from that found by Strohmeier et al. (2002), who observed an increase in Bax protein expression in BT-20 cell lines after a 24-hour treatment with tamoxifen.

It is possible that Bax protein expression has not been altered in this study due raloxifene has been used for a short period of time, nevertheless some authors such as Veronese et al. (1998), evaluating the effect of tamoxifen on the adjuvant treatment of 58 patients with invasive breast cancer for a prolonged period of 5 years also failed to find any difference in Bax protein expression. However, these authors did find an association between increased Bax protein expression and axillary lymph node involvement. Another study, evaluating the prognostic value of Bax in 145 women over 70 years of age with breast cancer, undergoing definitive surgical treatment, followed by adjuvant treatment with tamoxifen for up to 1 year, observed no relationship between Bax expression and disease-free survival (Daidone, 2000). Such findings were similar to those recently reported by Cannings et al. (2007).

Few in vitro studies have assessed Bax protein expression in breast cancer cell lines treated with tamoxifen (Salami et al. 2003, Kim et al. 2005). However, no in vitro or in vivo study has assessed the effect of raloxifene on Bax protein in breast cancer cells. A study using the Western blot technique evaluated the effect of raloxifene on Bax expression in cell lines of prostate and bladder cancer cells and observed no change in Bax protein expression (Kim et al. 2002). More recently, Furtado-Veloso et al (2008) assessed the effect of 60 mg of raloxifene administered for 22 days on Bax protein expression in normal breast tissue adjacent to fibroadenoma in premenopausal women and found no significant alteration in the biomarker after treatment with this type of SERM. The findings of those studies, despite the fact that they were in different situations, corroborate the findings of the present study.

Studies have shown the intense antiproliferative effects of SERMs on breast cancer cells (Johnston et al. 1994, Makris et al.1998, Clarke et al. 1993). Since cell proliferation and apoptosis are phenomenon well linked, possibly sharing common genic modulation, such as that promote by gene *c-myc* (Evan et al. 1994), and thus the inhibition of proliferative activity promoted by raloxifene, as demonstrated in some studies (Da Silva et al. 2006, Da Silva et al. 2009b), may interfere in the apoptotic process, leading to the results found in the present study.

In conclusion, raloxifene did not significantly alter Bax protein expression in estrogen receptor-positive invasive ductal carcinoma in postmenopausal women, when the drug was administered at a dose of 60 mg for 28 days. However, further studies are needed to evaluate the role of Bax as a prognostic and therapeutic biomarker when

SERMs are used, as well as its interaction with other proteins involved in the apoptotic phenomenon.

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Table I. Percentage of breast cancer cases with Bax-positive cells prior to and following raloxifene treatment.

	Post-Treatment		
Pre-Treatment	Negative	Positive	Total
Negative	9	0	9 (45%)
Positive	2	9	11 (55%)
Total	11 (55%)	9 (45%)	20 (100%)

There was no statistically significant alteration in Bax expression following raloxifene treatment ($p = 0,479$).

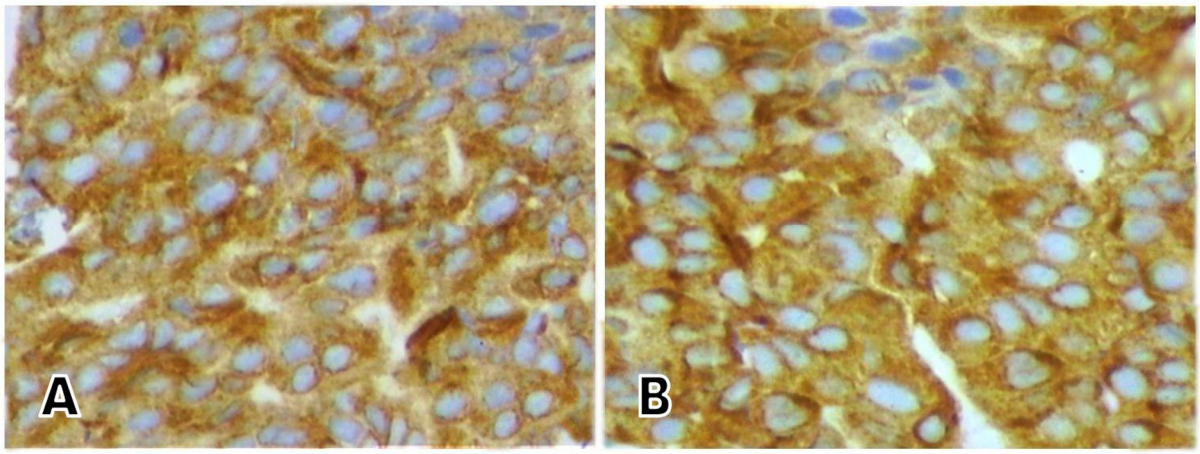

Figure 1: Photomicrograph of a histological section of breast cancer tissue sample from patient # 17, showing that the concentration of cells stained brown by anti-Bax antibody in the tumor sample obtained before raloxifene treatment (A) was similar to the concentration of cells positive for Bax expression in the sample collected after raloxifene treatment (B) (original magnification of 400X).

6. CONCLUSÕES

Artigo 1

O raloxifeno reduziu significantemente a expressão do antígeno Ki-67 e aumentou a expressão do antígeno Bcl-2 no carcinoma ductal infiltrante receptor de estrógeno positivo de mulheres pós-menopausadas.

Artigo 2

O raloxifeno não alterou significantemente a expressão da proteína Bax no carcinoma ductal infiltrante receptor de estrógeno positivo de mulheres pós-menopausadas.

7. CONSIDERAÇÕES FINAIS

No presente estudo o raloxifeno, quando administrado na dose de 60 mg via oral por 28 dias, reduziu significantemente a expressão da proteína Ki-67 e aumentou a expressão da proteína Bcl-2, contudo não alterou significantemente a expressão da proteína Bax no carcinoma ductal infiltrante receptor de estrógeno positivo de mulheres pós-menopausadas.

O Ki-67 e o Bcl-2 se mostraram promissores biomarcadores de resposta à terapia endócrina com raloxifeno, sofrendo alterações precocemente após administração da droga, o mesmo não observado em relação à proteína Bax, talvez porque a alteração deste biomarcador requeira uma exposição ao raloxifeno por um período de tempo mais prolongado.

Levando-se em consideração que o raloxifeno reduz a incidência de câncer invasivo de mama em proporção similar ao tamoxifeno, sem os efeitos adversos deste SERM no endométrio, tendo sido recentemente aprovado na quimioprevenção do câncer de mama em mulheres de alto risco para esta neoplasia, os resultados do presente estudo, ao mostrar que a droga reduziu a atividade proliferativa e aumentou a expressão do Bcl-2, são favoráveis à utilização do raloxifeno na endocrinoterapia do câncer de mama, fim para o qual ele ainda não foi aprovado. Contudo, mais estudos são necessários para avaliar o verdadeiro papel do raloxifeno no tratamento endócrino do câncer de mama.

8. ANEXOS

Anexo A: Termo de Consentimento Livre e Esclarecido

UNIVERSIDADE FEDERAL DO PIAUÍ
CENTRO DE CIÊNCIAS DA SAÚDE
DEPARTAMENTO MATERNO-INFANTIL

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Você está sendo solicitado para autorizar uma pesquisa. Você precisa decidir se quer autorizar ou não. Por favor, não se apresse em tomar a decisão. Leia cuidadosamente o que se segue e pergunte ao responsável pelo estudo sobre qualquer dúvida que tiver. Este estudo está sendo conduzido por BENEDITO BORGES DA SILVA. Após ser **esclarecida** sobre as informações a seguir, no caso de autorizar este estudo, assine este documento, que está em duas vias. Uma delas é sua e a outra é do pesquisador responsável. Em caso de recusa você não será penalizada de forma alguma. Em caso de dúvida você pode procurar o Comitê de Ética em Pesquisa – UFPI - Campus Universitário Ministro Petrônio Portella - Bairro Ininga Centro de Convivência L09 e 10 - CEP: 64.049-550 - Teresina – PI tel.: (86) 3215-5734 - email: cep.ufpi@ufpi.br web: www.ufpi.br/cep.

ESCLARECIMENTO SOBRE A PESQUISA:

Título do Projeto: "**EXPRESSÃO DOS ANTÍGENOS KI-67 E BCL-2 NO CARCINOMA DE MAMA DE MULHERES TRATADAS COM RALOXIFENO**".

Pesquisador Responsável: Benedito Borges da Silva.

Telefone para contato: (086) 3221-3040 (ramal 138).

♦ O presente estudo trata-se de um ensaio clínico, isto é, um estudo experimental, a ser realizado no Hospital Getúlio Vargas (HGV).

♦ O estudo envolverá cerca de 30 pacientes do sexo feminino pós-menopausadas, atendidas no Setor de Mastologia da Clínica Ginecológica do Hospital Getúlio Vargas da Universidade Federal do Piauí no período de março de 2009 a março de 2010, com diagnóstico de carcinoma ductal invasivo de mama.

♦ As pacientes tomarão um comprimido de raloxifeno na dose de 60 mg ao dia, por via oral, iniciado logo após a realização da biópsia confirmatória de câncer de mama, durante 28 dias, após o qual será realizada nova biopsia por ocasião do tratamento cirúrgico definitivo, que será cirurgia conservadora ou mastectomia radical, dependendo de cada caso.

♦ O raloxifeno é uma droga comercializada com o nome de Evista® para prevenção e tratamento da osteoporose. Tem como principais efeitos benéficos o fortalecendo dos ossos e a melhora dos níveis de colesterol das usuárias. Entre os efeitos colaterais relatados temos enjôos, vômitos, dor de estômago, onda de calor, câimbras e trombose venosa, esta de ocorrência bastante rara (três mulheres para cada 10 mil usuárias da medicação por ano).

♦ Este estudo tem por objetivo saber se o raloxifeno reduz a proliferação tumoral e se tem influencia benéfica sobre a morte das células tumorais.

♦ Garantia de acesso: em qualquer etapa do estudo, você terá acesso ao profissional responsável pela pesquisa para esclarecimento de eventuais dúvidas. O pesquisador responsável é o Sr. Benedito Borges da Silva, que pode ser encontrado no endereço: Av, Frei Serafim, 2280, Centro, Teresina-PI, Clínica Ginecológica do Hospital Getúlio Vargas, Telefone (086) 3221-3040 ramal 138. Se você tiver alguma consideração ou dúvida sobre a ética da pesquisa, entre em contato com o Comitê de Ética em Pesquisa – UFPI - Campus Universitário Ministro Petrônio Portella - Bairro Ininga Centro de Convivência L09 e 10 - CEP: 64.049-550 - Teresina – PI tel.: (86) 3215-5734 - email: cep.ufpi@ufpi.br web: www.ufpi.br/cep.

♦ O período de participação será de março de 2009 a março de 2010, durante o qual o participante terá o direito de retirar o seu **consentimento** a qualquer momento.

Pesquisador

CONSENTIMENTO

Eu, _____abaixo assinado, concordo em participar do estudo intitulado “EXPRESSÃO DOS ANTÍGENOS KI-67 E BCL-2 NO CARCINOMA DE MAMA DE MULHERES TRATADAS COM RALOXIFENO”. Declaro que tive pleno conhecimento das informações que li ou que

foram lidas para mim**.** Discuti com o Sr. Benedito Borges da Silva sobre a minha decisão em participar nesse estudo. Ficaram claros para mim quais são os propósitos do estudo, os procedimentos a serem realizados, a ausência de riscos, as garantias de confidencialidade e de esclarecimentos permanentes. Ficou claro também que minha participação é isenta de despesas e que tenho garantia do acesso à pesquisa. Concordo, voluntariamente, em participar deste estudo e poderei retirar o meu consentimento a qualquer momento, antes ou durante o mesmo. A retirada do consentimento da participação no estudo não acarretará penalidades ou prejuízos nessa Instituição ou Serviço.

Teresina, ____ de _____ de _____.

Nome e Assinatura do sujeito ou responsável:

_____

_____

Benedito Borges da Silva

Pesquisador Responsável

Presenciamos a solicitação de consentimento, o esclarecimento sobre a pesquisa e o aceite da paciente em participar do estudo acima descrito:

Testemunhas (não ligadas à equipe de pesquisadores):

Nome: _____

Assinatura: _____

Nome: _____

Assinatura: _____

Observações Complementares:

Se você tiver alguma consideração ou dúvida sobre a ética da pesquisa, entre em contato:
Comitê de Ética em Pesquisa – UFPI - Campus Universitário Ministro Petrônio Portella - Bairro Ininga
Centro de Convivência L09 e 10 - CEP: 64.049-550 - Teresina - PI
tel.: (86) 3215-5734 - email: cep.ufpi@ufpi.br web: www.ufpi.br/cep

Anexo B: Ficha de Coleta de Dados

FICHA CLÍNICA DE MASTOLOGIA

Data _____/_____/_____

Identificação:
Numero do Prontuário:_____Profissão_____
Endereço_____
Cidade_____UF_____CEP_____Fone:_____
Idade _____Escolaridade _____Estado Civil_____
Raça _____Cor_____
Queixas

Mama Direita
Nódulo_____
Dor_____
Fluxo_____
Inflamação_____
Trauma_____
Axilas e FossasClaviculares_____
Outros_____

Mama Esquerda
Nódulo_____
Dor_____
Fluxo_____
Inflamação_____
Trauma_____
Axilas e Fossas Claviculares_____
Outros_____

Antecedentes Pessoais: _____

Antecedentes Familiares: _____

Antecedentes Ginecológicos
Menarca _____ G _____P_____A_____ Idade do 1º. Parto _____
Menopausa _____TH_____
Alcolismo_____
Tabagismo _____
Cirurgias_____

Estadiamento Clínico_____
Conduta_____

Anexo C: Parecer do Comitê de Ética em Pesquisa da UFPI

MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DO PIAUÍ
COMITÊ DE ÉTICA EM PESQUISA
Campus Universitário Ministro Petrônio Portela, Bairro Ininga, Teresina, Piauí, Brasil; CEP 64049-550
Telefones: (86) 3215-5734 Fax (86) 3215-5560

Teresina, 23 de Maio de 2006

Ao(à):
Sr(a). PEDRO VITOR LOPES COSTA
Mestrado em Ciências da Saúde
Centro de Ciências da Saúde - CCS

Senhor(a) Pesquisador(a),

O Comitê de Ética em Pesquisa (CEP), da Universidade Federal do Piauí analisou, de acordo com os requisitos da Resolução CNS 196/96, que trata das "diretrizes e normas envolvendo seres humanos", o protocolo de pesquisa encaminhado por V.sa., intitulado "QUANTIFICAÇÃO DA ANGIOGÊNESE NO CARCIOMA DE MAMA DE MULHERES TRATADAS COM RALOXIFENO" o qual teve parecer "aprovado" sob o número 022/2006. Ressaltamos que o parecer consubstanciado emitido na reunião encontra-se arquivado para eventuais consultas.

Atenciosamente

Profª. Drª. Regina Ferraz Mendes
Comitê de Ética em Pesquisa-UFPI
COORDENADORA

Anexo D: Carta de aceitação do artigo ¨Evaluation of Ki-67 and Bcl-2 antigen expression in breast carcinomas of women treated with raloxifene¨ no periódico *Cell Proliferation* (Fator de Impacto JCR 2007: 3,120)

Decision Letter (Cellprol-0646-09.R1)

From: sarrafc@wmin.ac.uk

To: beneditoborges@globo.com

CC:

Subject: Cell Proliferation - Decision on Manuscript ID Cellprol-0646-09.R1

Body: 15-Jun-2009

Dear Prof. da Silva:

It is a pleasure to accept your manuscript entitled "Evaluation of Ki-67 and Bcl-2 antigen expression in breast carcinomas of women treated with raloxifene" in its current form for publication in Cell Proliferation. The comments of the reviewer who finally refereed your manuscript are included at the foot of this letter.

Thank you for your excellent submission.

Please find attached an Exclusivity Licence Form (copyright assignment ctaaglobal form) for you to sign; it is part of the publication process. Please could you print it off and sign it where indicated, then post the hard copy to our Production Editor Ms Esther Chia, in Singapore. It has to go by mail, I'm afraid, as we must have the original signature. Ms Chia's details are:

Ms Esther Chia,
Production Editor

Journal Content Management
Wiley-Blackwell
Blackwell Publishing Services Pte Ltd
#05-01 Parkview Square
600 North Bridge Road
Singapore 188 778

Email: echia@wiley.com

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We look forward to your continued contributions to the Journal.

Yours sincerely,
Dr. Catherine Sarraf
Editor in Chief,
Cell Proliferation
sarrafc@wmin.ac.uk

Reviewers' Comments to Author:

Reviewer: 1
Comments to the Author
Authors have adequately completed the revision and the manuscript has now improved substantially. I have no other comments.

Date Sent: 15-Jun-2009

Anexo E: Carta de comprovação de submissão do artigo "The effect of raloxifene on Bax protein expression in breast carcinomas of postmenopausal women" no periódico *Biomarkers* (Fator de Impacto JCR 2007: 1,978)

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PEDRO VITOR LOPES COSTA <pvlcosta@ig.com.br>

Fwd: BiOMARKERS - Manuscript ID TBMK-2009-0149

1 mensagem

Benedito Borges da Silva <beneditoborges@globo.com> **17 de agosto de 2009 17:47**
Para: PEDRO VITOR LOPES COSTA <pvlcosta@ig.com.br>

---------- Forwarded message ----------
From: <alanpaine@hotmail.com>
Date: 2009/8/17
Subject: BiOMARKERS - Manuscript ID TBMK-2009-0149
To: beneditoborges@globo.com

17-Aug-2009

Dear Professor da Silva

Your manuscript entitled "The effect of raloxifene on Bax protein expression in breast carcinomas of postmenopausal women" has been successfully submitted online and is now being given full consideration for publication in BiOMARKERS.

Your manuscript ID is TBMK-2009-0149.

Please use the above manuscript ID in all future correspondence and especially in the subject line of any e-mails you may send or when calling the office for questions. If there are any changes in your street address or e-mail address, please log in to ScholarOne Manuscripts at http://mc.manuscriptcentral.com/tbmk and edit your user information as appropriate.

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Thank you for submitting your manuscript to BiOMARKERS.

Sincerely,

Editor-in-Chief
BiOMARKERS

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