RESEARCH ARTICLE

Expression of Bax and Bcl-2 in Tumour Cells and Blood Vessels of Breast Cancer and their Association with Angiogenesis and Hormonal Receptors

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Abstract

A total of 96 cases of invasive breast ductal carcinoma were examined for immunohistochemical expression of Bax and Bcl-2 in the epithelial tumor cells and endothelial cells of the blood vessels. We also investigated the association between both proteins in the epithelium in relation to tumor characteristics such as tumor size, grade, lymph node involvement, microvessel density (MVD), hormonal receptors expression and c-erbB-2 overexpression. Bax expression showed a significant association between tumor and endothelial cells (p<0.001) while Bcl-2 expression in tumor cells was inversely associated with that in the endothelial cells (p<0.001). Expression of Bcl-2 in tumor cells was strongly associated with expression of estrogen and progesterone receptors (p=0.003 and p=0.004, respectively). In addition, intratumoral MVD was significantly higher than peritumoral MVD (p<0.001) but not associated with Bax or Bcl-2 expression and other tumor characteristics. We concluded that the number of endothelial cells undergoing apoptosis was in direct linkage with the number of apoptotic tumor cells. Anti-apoptotic activity of the surviving tumor cells appears to propagate cancer progression and this was influenced by the hormonal status of the cells. Tumor angiogenesis was especially promoted in the intratumoral region and angiogenesis was independent of anti-apoptotic activity.

Keywords: Apoptosis - angiogenesis - breast cancer cells - blood vessels - hormonal receptors

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Introduction

Breast cancer is a heterogenous disease with variable biological and clinical characteristics. In understanding the mechanism of breast cancer development and progression, the anti-apoptotic and angiogenic activities of the tumor cells have been studied and these include the study of pro-apoptotic Bax and anti-apoptotic Bcl-2 gene expressions. Apoptosis inhibition depends partly on the balance of expression between Bcl-2 and Bax gene, with most studies found overexpression of Bcl-2 in the tumor cells correlates with poor patient prognosis and an association with p53 mutations and estrogen receptor expressions (Yang et al., 1999; Baccouche et al., 2003; Sirvent et al., 2004; Linjawi et al., 2004). However, Bax expression in tumor cells showed no correlation with prognosis (Veronese et al., 1998; Yang et al., 1999; Baccouche et al., 2003).

Angiogenesis has been demonstrated to be a very important mechanism for breast cancer cell survival and progression (Folkman, 2003). It involves the process of endothelial cell activation, degradation of the basement membrane and extracellular matrix, with migration and proliferation of endothelial cells. It has been associated with overexpression of estrogen and progesterone receptors (Vamesu, 2008), progression from ductal carcinoma in-situ (Vogl et al., 2005), metastasis (Weidner et al., 1991) and poor prognosis (Karelia et al., 1997; Jan-Show et al., 1998; Nazan et al., 2002).

Studies associating tumor apoptosis and angiogenesis reported that the angiogenic switch in a tumor is followed by decreased in apoptosis of the tumor cells (Holmgren et al., 1995; Hanahan et al., 1996; Carmeliet et al., 1998; Merja et al., 1999). However, no study has so far looked at this association in the tumor blood vessels.

Therefore, we analyzed the expressions of Bax and Bcl-2 in the breast cancer cells as well as simultaneous analysis of both expressions in endothelial cells of the blood vessels that supply the cancer cells. We further evaluated their relationship with microvessel density and other established prognostic factors.

Materials and Methods

Breast Cancer Specimens

Paraffin-embedded breast tumor tissue blocks of 96 women diagnosed as breast cancer patients at Universiti Sains Malaysia Hospital in Kelantan, Malaysia were retrieved for the study. Information regarding age, sex, tumor size, and lymph node status were obtained from patients' clinical record and Pathology Registry. Ethical clearance for the study was obtained from The Ethical Committee, Universiti Sains Malaysia.

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All tumors were infiltrating ductal carcinoma Not Otherwise Specified (NOS) in which 79 cases were obtained from mastectomy specimen, while the rest were either from wide excision or lumpectomy specimens. The tissue slides were retrieved to review the histology grade of the tumors. The tumors were graded according to the modified Scarff-Bloom Richardson grading system (Frierson et al., 1995). The slides of immunohistochemical stain for overexpressions hormonal receptors and c-erb-B2 were retrieved and reviewed. Scoring was based on the Allred Scoring System and Dako HercepTest Protocol System respectively without prior knowledge of the score in the initial report (Allred et al., 1998; DAKO HercepTest, 1999; Qureshi and Pervez, 2010).

Immunohistochemical staining for Bax, Bcl-2 and CD 34

Four μm thick, formalin-fixed, paraffin-embedded sections were immunostained using primary antibodies to Bax, Bcl-2 and CD 34 as detailed in Table 1.

Sections were dewaxed in xylene and rinsed in graded alcohols. Endogenous peroxidase was blocked by incubation in 3% hydrogen peroxidase for 15 minutes, followed by rinsing in distilled water. The sections were subsequently subjected to antigen retrieval by immersing in citrate buffer (pH 6) followed by heating in a pressure cooker (DAKO®, Denmark), and washing in tris buffered saline (TBS).

Nonspecific staining was blocked by treating with blocking serum (normal rabbit serum) for 20 minutes. Following an incubation of 30 minutes for all the primary antibodies (except Bax which was incubated overnight), washing with TBS and application of horse radish peroxidase (HRP) polymer for 30 minutes were done with subsequent repeat washing in TBS. Di-amino benzidine (DAB) was then applied to the sections and incubated for 3 minutes, followed by counterstaining with haematoxylin.

Sections without the primary antibodies staining served as negative controls and these were run with each batch of staining, together with positive controls for each antibody. Lymph node tissue of Hodgkin Lymphoma was used in positive control for Bax and tissues from tonsils for Bcl-2 and CD34.

Staining Characteristics and Assessment of Staining

Tissue sections immunostained for Bax and Bcl-2 were assessed for apoptotic activity. The intensity of cytoplasmic staining reactions was graded into four qualitative score groups: 1 = weak staining intensity; 2 = moderate staining intensity; 3 = strong staining intensity; and 4 = very strong staining intensity. The immunostaining results were quantified as follows: 0 = no positive staining; 1 = <25% of tumor cells or vessels showing cytoplasmic staining positivity; 2 = 25 – 50 % of tumor cells or vessels showing cytoplasmic positivity; 3 = 50 – 75 % of tumor cells or vessels showing cytoplasmic positivity; 4 = >75 % of tumor cells or vessels showing cytoplasmic positivity. A combined score for Bax and Bcl-2 immunostaining was obtained by adding the qualitative and quantitative scores and the summations were grouped into three scores: score = 0, no immunoreactivity; score =1-4, weak immunoreactivity; and score = 5-8, strong immunoreactivity (Merja et al., 1999).

The evaluation of immunohistochemical expression of Bax and Bcl-2 were done under x400 magnification using an Olympus® CX31 light microscope. A total of 100 cells were counted for each scoring, with the scoring repeated twice and the means calculated.

Counting of microvessel density (MVD) was based on CD34 positive staining blood vessels. Areas with the highest level of angiogenesis were identified first by scanning the entire tumor section at lower magnification (x40 and x100), followed by microvessels counting at magnification of x200. An ocular grid was used to facilitate the counting procedure by providing a framework for systematic counting. A microvessel was defined as any staining of the endothelial cells or cell clusters that clearly appeared separated from adjacent microvessels. Neither vessel lumen nor the presence of red blood cells were required to define a microvessel. Three x200 magnification fields were chosen among areas with highest microvessel density appearance (hot spots) and the mean density were calculated from the counting results of the three hot spots (Jan-Show et al., 1998 and Nazan et al., 2002). Peritumoral area was defined as an area with vessels within one high power field (x400), or 5 mm around the tumor cell-occupied area, or away from the advancing edge of the tumor mass (Giorgadze et al., 2005). All the slides evaluated for Bax, Bcl-2 and MVD were done without prior knowledge of the clinical data of the patients.

Data Analysis

Data entry and analysis was done using SPSS Version 12.0.1 (Spss Inc, 2003). All continuous variables were described using mean (SD) and categorical data as frequency (%). Paired t-test was used to compare the expression of Bax and Bcl-2 between those in tumor cells and those in intratumoral blood vessels, as well as to compare the microvessel density in intratumoral and peritumoral regions.

The association between the Bax and Bcl-2 expression was assessed using the Mc-Nemar test for categorical data. The correlation between intratumoral Bax and Bcl-2 expressions and microvessel density was determined by using Pearson correlation coefficient test. Associations between each tumor characteristic with regards to Bax, Bcl-2 and MVD were analyzed using chi-square test for categorical data and independent t-test for numerical data.

Table 1. Primary Antibodies Used in the Study

Antibody	Source	Type	Pretreatment	Dilution
Bcl-2	DAKO®	Mouse Monoclonal	Pressure cooking 3 minutes	1:50
Bax	DAKO®	Rabbit Polyclonal	Pressure cooking 3 minutes	1:200
CD34	DAKO®	Mouse Monoclonal	Pressure cooking 3 minutes	1:100

Table 2. Tumor Characteristics Among Study Subjects (N=96)

Characteristics		Frequency	Percentage
Tumor size	< 2cm	6	6.3
	2 – 5 cm	48	50.0
	> 5 cm	42	43.7
LN involvement	Yes	46	47.9
	No	42	43.8
	Unknown	8	8.3
Tumor grade	I	18	18.8
	II	32	33.3
	III	46	47.9
ER	Positive	42	43.8
	Negative	54	56.2
PR	Positive	46	47.9
	Negative	50	52.1
c-erbB-2	Negative	42	43.8
	+	12	12.5
	++	20	20.8
	+++	22	22.9

Table 3. Comparison Bax in Tumor Cells (TC) and Bax in Intratumoral Blood Vessels (BV) [score] Among Study Subjects (N=96)

	Mean (SD)	Mean diff. (SD)	t-stat	95% CI[a]	Df[b]	p-value[c]
Bax-TC	5.9 (1.8)	3.2 (1.6)	19.743	2.90, 3.54	95	<0.001
Bax-BV	2.7 (1.8)					

[a]CI, Confidence Interval; [b]df, degree of freedom; [c]paired t- test

Results

Clinical and tumor characteristics

The mean age of the patients at the time of breast cancer diagnosis was 49.9 years. The youngest was a 28-years old while the oldest was 82. One third of the patients (33.3%) lie within the 40 to 49 age range. Tumor characteristics are listed in Table 2. All the patients had invasive ductal carcinoma (IDC) with about half of them with Grade 3 IDC (46/96), whereas 33.3% (32/96) and 18.8% (18/96) had Grade 2 and Grade 1 IDC, respectively.

The estrogen receptor (ER) and progesterone receptor (PR) status of the tumor was reviewed for all cases. More than half (56.2%) were ER negative; with the rest (42/96) positive. PR positivity was seen in 47.9% (46/96) and negativity in 52.1% (50/96). Nearly half of the IDC cases (43.8%) were clear cut negative for c-erbB-2. Twelve cases (12.5%) showed only 1+ staining intensity and these were also considered as negative. Forty-two positive cases had a 2+ score (20.8%) and 3+ score (22.9%).

Expression of Bax (pro-apoptotic) protein

Stronger expressions of Bax (p<0.001) was found in tumor cells (TC) when compared to those expressed in intratumoral blood vessels (BV) (Figure 1 and Table 3).

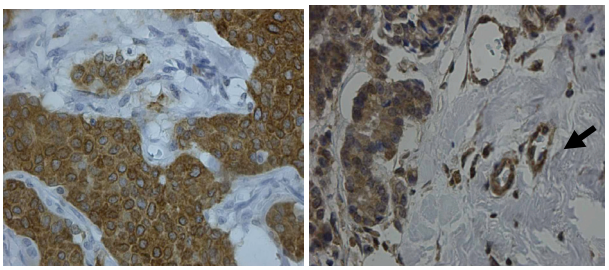

Figure 1. Bax Cytoplasmic Positivity. a) Tumour cells; b) Endothelial cells (x400)

Table 4. Association Between Bax in Tumor Cells (TC) and Bax in Intratumoral Blood Vessels (BV) [Category] Among Study Subjects (N=96)

Variable (Immunoreactivity)	Bax-BV Negative Freq(%)	Bax-BV Positive Freq(%)	p-value[c]
Bax-TC			<0.001
Negative	3(3.1)	0(0.0)	
Positive	18(18.9)	75(78.1)	

[c]Mc-Nemar test

Table 5. Comparison Bcl-2 in Tumor Cells (TC) and Bcl-2 in Intratumoral Blood Vessel (BV) [score] Among Study Subjects (N=96)

	Mean (SD)	Mean diff. (SD)	t-stat	95% CI[a]	Df[b]	p-value[c]
Bcl-2 TC	4.1(3.2)	3.2 (3.2)	9.710	2.54, 3.85	95	<0.001
Bcl-2 BV	0.9(1.4)					

[a]CI, Confidence Interval; [b]df, degree of freedom; [c]paired t- test

Table 6. Association Between Bcl-2 in Tumor Cells(TC) and Bcl-2 in Intratumoral Blood Vessels(BV) [Category] Among Study Subjects (N=96)

Variable (Immunoreactivity)	Bax-BV Negative Freq(%)	Bax-BV Positive Freq(%)	p-value[c]
Bcl-2-TC			<0.001
Negative	21(21.9)	9(9.4)	
Positive	43(44.8)	23(24.0)	

[c]Mc-Nemar test

Some 78.1% of cases showed simultaneous strong expression in TC and BV, whereas only 3.1% showed no simultaneous expression of Bax. 18.9 percent showed strong positivity of Bax in TC with no expression of Bax in BV. There was a significant association of Bax expression in both groups (p<0.001) (Table 4).

Expression of Bcl-2 (anti- apoptotic) protein

Similar to expression of Bax, the expression of Bcl-2 in TC was comparatively stronger than that in the BV (p<0.001) (Table 5). Some 24% of the cases had simultaneous strong expression in both TC and BV whereas negativity in both was found in 21.9%. Forty-four point eight percent showed positivity in TC without positivity in BV, and 9.4% showed positivity in BV but not in TC. There was a significant association of Bcl-2 expression between both groups (p<0.001) (Table 6).

Microvessel density (MVD) in intratumoral and peritumoral area

Microvessel density was found to be higher in intratumoral (IT) region (mean ± SD = 54.1 ± 16.2) as compared to microvessel density in peritumoral (PT) region (mean ± SD = 46.3 ± 15.6). There is significance difference (p<0.001) in microvessel density count between both IT region and PT region (Table 7).

Correlation between intratumoral MVD with Bcl-2 and Bax expressions of tumor cells

There is no significant (linear) correlation between

Table 7. Comparison of MVD in Intratumoral (IT) and Peritumoral (PT) Regions Among Study Subjects (N=96)

	Mean (SD)	Mean diff. (SD)	t-stat	95% CI[a]	Df[b]	p-value[c]
MVD IT	54.1(16.2)	7.8 (19.0)	4.013	3.94, 11.66	95	<0.001
MVD PT	46.3 (15.6)					

[a]CI, Confidence Interval; [b]df, degree of freedom; [c]paired t- test

Table 8. Association Between Expression of Bcl-2 in Tumor Cells (TC) with Tumor Characteristics

Variable		Bcl-2-TC		p-value[c]
		Negative Freq,%	PositiveFreq,%	
Tumor size				0.712
	< 2cm	3(3.1)	3(3.1)	
	2 – 5 cm	21(21.9)	27(28.1)	
	> 5 cm	22(22.9)	20(20.8)	
LN involvement				
	Yes	21(21.9)	25(26.0)	0.913
	No	21(21.9)	21(21.9)	
	Unknown	4(4.2)	4(4.2)	
Tumor grade				0.241
	I	8(8.3)	10(10.4)	
	II	12(12.5)	20(20.8)	
	III	25(27.1)	20(20.8)	
ER	Positive	13(13.5)	29(30.2)	0.003
	Negative	33(34.4)	21(21.9)	
PR	Positive	15(15.6)	31(32.3)	0.004
	Negative	31(32.3)	19(19.8)	
c-erbB-2				0.051
	Negative	14(14.6)	28(29.2)	
	+	7(7.3)	5(5.2)	
	++	10(10.4)	10(10.4)	
	+++	15(15.6)	7(7.3)	

[c]Pearson Chi-square test

MVD and expression of Bcl-2 and Bax in tumor cells score (p= 0.594 and p= 0.690, respectively). The observed r (Pearson's Correlation Coefficient) is -0.06 and -0.04, which suggests no correlation.

Relationship between tumor cell expression of Bcl-2 and Bax with MVD and tumor characteristics

No association was noted between the expression of Bcl-2 in tumor cells with microvessel density, tumor size, lymph nodes involvement, tumor grade and overexpression of c-erbB-2. However, there was significant association between expression of Bcl-2 in tumor cells with oestrogen and progesterone receptors with p-value of 0.003 and 0.004 respectively (Table 8).

There was no association between expression of Bax with microvessel density, tumor size, lymph node involvement, tumor grade, oestrogen receptor, progesterone receptor and overexpression of c-erbB-2. There was also no association between microvessel density with the above mentioned tumor characteristics.

Discussion

Apoptosis is a programmed cell death that was initially described as shrinkage necrosis by Kerr et al (Kerr et al., 1972) in their study of cell death in liver and adrenal cells. It was later in 1972 when the term 'apoptosis' was coined for this mode of cell death (White, 1996). Apoptosis is a highly controlled process that occurs both in normal and pathological conditions, with malignant tumors being part of the latter (White, 1996; Kahlos et al., 2000; Hope et al., 2001; Ray et al., 2002; Harada et al., 2004). The balance in the expression of Bax pro-apoptotic protein and Bcl-2 anti-apoptotic protein is partly responsible for apoptosis inhibition; and this has been shown to be important in breast cancer pathogenesis (White, 1996; Merja et al., 1999). Researchers had shown that apoptosis increases in malignant tumor (Kahlos et al., 2000; Ray et al., 2002). The increased apoptosis, together with tumor cell proliferation are most likely to occur in higher grade tumor and in recurrent breast cancer; a fact that could be used to predict a poor prognosis in breast cancer patients (Krajewski et al., 1999; Lipponen et al., 1999; Merja et al., 1999).

Apoptosis of endothelial cells is also known to occur in both normal and pathological conditions (Djonov et al., 2001; Affara et al., 2007; Hasnan et al., 2010); the apoptosis plays an important role in blood vessel development, homeostasis and remodelling (Affara et al., 2007). The apoptosis of breast cancer cells and endothelial cell of the supplying blood vessels was investigated in our study. The results showed a direct association and an increased in apoptotic activity in both the tumor cells and the endothelial cells (p<0.001). Interestingly, the anti-apoptotic activity (based on expression of Bcl-2) of the endothelial cells is inversely associated with those of the tumor cells (p<0.001). In other words, there was a decrease in anti-apoptotic activity of the endothelial cells in contrast to an increased activity in the tumor cells.

Most endothelial cells in adult blood vessels are relatively quiescent and resistant to apoptosis. In contrast, destabilized blood vessels that occurs in tumors results in endothelial cell apoptosis. The interaction of tumor cells with host blood vessels is associated with angiogenic stimuli such as vascular endothelial growth factor (VEGF), Ang-1 and Ang-2. VEGF produces immature, leaky and hemorrhagic vessels. Ang-1 on the other hand makes vessels resistant to leakage, stabilizes the vessel walls and maximizes the interaction between endothelial cells and the surrounding supporting cells and matrix (Thurston et al., 1999). Ang-2 however, destabilizes blood vessels by blocking Ang-1 and its Tie2 receptors (Maisonpierre et al., 1997). The current opinion among researchers nowadays believe that neoplasia starts off as well-vascularized small tumors (Holash et al., 1999; Yancopoulos et al., 2000). Tumors cells initially home in and grow by co-opting the existing available blood vessels. As a defence mechanism, the host blood vessel regresses when sensing inappropriate co-option resulting in tumor starvation, leading to avascularity and hypoxia of the tumor. This phenomenon is associated with endothelial Ang-2 production that causes vessel destabilization, apparently leading to vessel regression and death via apoptosis (Yu and Stamenkovic, 2001). It is possible that the direct contact of tumor cells with the host blood vessels wall impairs endothelium integrity, which also lead to endothelial cell apoptosis (Kebers et al., 1998).

In our study, the higher expression of Bax in endothelial cells indicates higher apoptotic activity and subsequent tumor cells apoptosis. We believe that the

apoptosis in the endothelial cells of intratumoral blood vessels may be the triggering factor that selectively causes an increase apoptosis in tumor cells.

The tumor under hypoxic condition secretes an abnormal burst of VEGF, which stops the regression of destabilized co-opted blood vessels and promotes robust new angiogenesis (Yancopoulos et al., 2000). The mechanism described is most probably achieved by the surviving subclones; not by the tumor cells per se. We believe that the surviving subclones are the one that has increased overexpression of Bcl-2 that make them resistant to apoptosis (Carmeliet et al., 1999; Zheng et al., 2000), and it is these surviving clones that later develop and progress under the influences of tumor-associated VEGF and Ang-2.

We did not find any significant association between tumor cells expression of Bcl-2/Bax and intratumoral microvessel density. The microvessels were detected using anti- CD34 which was shown to be a reproducible and reliable method of cancer neovascularization, compared to other techniques such as anti-CD31 or anti-Factor VIII-related antigen immunostaining (Weidner et al., 1991). Moreover, CD34 also identifies the small caliber microvessel more efficiently than vWF (Tenderenda et al., 2001). Among women with invasive cervical cancer, Tjalma et al. (2001) did not find any significant association between Bax and microvessel density, but Bcl-2 expression was found to be related to vessel density (p=0.015). Perrone et al. (2004) found that Bcl-2 expression was positively correlated with microvessel density in colon cancer (p= 0.009). However, they did not do study on Bax expression. We postulate that the possible reasons for variability of findings by the different researchers could be attributed to the nature of the tumor studied, stages of microvessel development and the method used to define and detect the microvessels.

We also did not demonstrate any significant association between microvessel density (intratumoral) with tumor size, lymph nodes involvement, tumor grade, hormonal status (estrogen and progesterone receptors) and overexpression of c-erbB-2. Axelsson et al. (1995) visual assessment of angiogenesis using anti-Factor VIII-associated antigen found no correlation with lymph node status or any other breast cancer parameter.

Our study showed significant association between the expressions of Bcl-2 with hormonal receptors. We found that the estrogen and progesterone receptors status were highly associated with Bcl-2 expression (p=value 0.003 and p=0.004, respectively). The finding is supported by other studies which also found significant association between the expression of Bcl-2 with estrogen and progesterone receptors status (Kobayashi et al., 1997; Krajewski et al., 1999; Merja et al., 1999; Rochaix et al., 1999; Al-Maoundhri et al., 2003).

The actions of Bcl-2 generally underlie the well known survival functions of hormones and growth factors. In breast epithelial cells, estrogen stimulation leads to up regulation of Bcl-2 and resistance to apoptosis (Soini et al., 1998). In vitro study by Gompel et al. (2000) showed that oestradiol could decrease apoptosis in human breast epithelial cells.

In conclusion, there is a direct association between apoptosis of the endothelial cells of the supplying blood vessels and apoptosis of the tumor cells. It suggests that endothelial cell apoptosis influences and may triggers the corresponding tumor cell apoptosis.bSurviving tumor cells show increased anti-apoptotic activity that contributes to the cancer progression, with the expressions of estrogen and progesterone receptors as associating factors. Therefore, the survival and progression of viable cancer cell subclones are dependent on the cells' ability to avoid apoptosis, and also by responding well to sustaining hormones. The angiogenesis of breast cancer that occurs independently of anti-apoptotic activities in the tumor cells or the endothelial cells of the tumor blood vessels, indicates that angiogenic activity of tumor microenvironment does not influence the selection of the surviving tumor subclones.

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