RESEARCH ARTICLE

Different Prognostic Factors Correlate with Bcl-2 Expression among Triple Negative and Non-Triple Negative Breast Cancers

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Abstract

Background: Prognostication of breast cancer using clinico-pathologic variables, although useful, remains imperfect. Recent research has focused on finding new markers of prognosis using gene expression profiling. Panels of proteins assessed by immunohistochemistry might also be useful in this regard. This study focused on Bcl-2 protein expression in triple-negative (TNBC) and non- triple-negative breast cancer (non-TNBC) with correlation to clinico-pathologic variables. Materials and methods: We analyzed Bcl-2 expression in 77 women with primary breast carcinoma divided into two groups; triple-negative and non- triple-negative according to expression of estrogen (ER), progesterone (PR) and human epidermal growth factor receptors (Her2/neu). Bcl-2 expression was assessed in relation to age, histo-pathological subtype, grade, nodal status and tumor size. Results: Bcl-2 was expressed in 74% of triple-negative breast cancers and 70% of non- triple-negative cancers. In TNBC, expression was significantly correlated with invasive ductal subtype, while in non-TNBC it was significantly correlated with age and negative nodal status. In both groups higher Bcl-2 expression associated with favourable prognostic factors in breast cancer, but no significant statistical correlations were found. Conclusions: Frequency of Bcl-2 expression does not differ between TNBC and non-TNBC, but different prognostic factors correlate with Bcl-2 in the two cases.

Keywords: Breast carcinoma - Bcl-2 expression - triple-negative - prognosis

Asian Pacific J Cancer Prev, **14 (2)**, 1037-1041

Introduction

Breast cancer is the most common malignancy among Women (Jemal et al., 2007). Based on histopathological characteristics, the World Health Organization has defined a wide range of invasive breast cancer subtypes (Tavassoli and Devilee, 2003). Unfortunately, this way of categorizing breast tumors fails to predict prognosis and treatment possibilities (de Ruijter et al., 2011). Recently, gene-expression analysis-based studies subdivided breast carcinomas into molecular subtypes (Perou et al., 2000; Sorlie et al., 2001). Furthermore, immunohistochemical studies analyzing protein expression has identified immuno-phenotypes of breast cancer identical to those derived from gene expression (Callagy et al., 2003; Abd El-Rehim et al., 2005). This has resulted in the recognition of the ''triple negative breast cancer'' (TNBC) (Van't et al., 2002; Sorlie et al., 2003; Paik et al., 2006), which accounts for about 15% of breast cancers (Kaplan et al., 2006). The term describes breast cancers that lack expression of the estrogen receptor (ER) and progesterone receptor (PR) and do not overexpress human epidermal growth factor 2 receptor (Her2/neu) protein (Ismail-Khan and Bui , 2010). TNBCs are biologically aggressive neoplasms with poor prognosis, frequent relapses and visceral metastasis (De Giorgi et al., 2007; Ismail-Khan and Bui, 2010).

To identify additional prognostic markers and improve risk stratification for breast cancer, further studies suggested combining protein markers used to define these molecular subtypes (Callagy et al., 2006; Nadler et al., 2008; Dawson et al., 2010). Indeed, they showed that only Bcl-2 added prognostic information independent of the other accepted prognostic factors (Callagy et al., 2006).

Bcl-2 (acronym for the B-cell lymphoma/leukemia-2 gene) was first discovered in B-cell malignancies (Tsujimoto and Croce, 1986). Specific translocation moves Bcl-2 gene from its normal location at 18q21 into the locus at 14q32, resulting in permanent activation of Bcl-2 gene and overproduction of Bcl-2 protein (Reed, 1994). Although Bcl-2 is an anti-apoptotic protein, high Bcl-2 expression in breast cancer, was linked to low-grade, slowly proliferating, estrogen receptor (ER), progesterone (PR) positive breast tumors and has been associated with improved survival (Lipponen et al., 1995; Bilalovic et al., 2004; Thomadaki et al., 2007; Trere et al., 2007).

Hence, Bcl-2 has two opposing activities, one (anti-apoptotic) that promotes tumorigenesis, and another (anti-proliferative), which is antitumorigenic (Reed et

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al., 1997; Zinkel et al., 2006; Subhawong et al., 2010), contrary to the above favourable prognostic data comes through laboratory-based data that suggest that Bcl-2 expression correlates with aggressive, prometastatic and chemotherapy resisting behavior in breast cancer (Kumar et al., 2000; Pinkas et al., 2004; Mimori et al., 2005; Subhawong et al., 2010). For this, the favourable prognostic data regarding Bcl-2 are difficult to reconcile with these prometastatic activities (Subhawong et al., 2010), and are insufficient for definitive conclusions to be drawn about the role of Bcl-2 as a predictive factor in breast carcinoma (Munster and Norton, 2001).

This study represents a comparison of Bcl-2 immunohistochemical expression in two main immunophenotypes of breast carcinoma: triple negative breast carcinomas (TNBC) and non-triple negative breast carcinomas (non-TNBC). In addition, it specifies which of the well accepted prognostic factors in breast cancer (such as patient's age, histopathological subtype, histological grade, and nodal status and tumor size) is correlated with Bcl-2 expression in each group using the standard statistical techniques

Materials and Methods

Seventy-seven cases of primary breast carcinomas were included in this study. All patients were females. The inclusion criteria were a histopathological diagnosis of invasive breast carcinoma and the availability of clinical data and paraffin-embedded tissue specimens. Patient's age and tumor size were retrieved from pathological reports. Routinely stained hematoxylin and eosin slides were reviewed by two pathologists and subtyped according to the WHO classification of breast cancer. Tumors were graded according to a modified Bloom–Richardson scoring system and axillary lymph node status was assessed.

Immunohistochemistry (IHC)

After deparaffinization and rehydration, 4-μm thick sections on coated slides were heat-pretreated in a citrate buffer (pH 7.3 at 92C) and immunostained using monoclonal primary antibodies against the following antigens: estrogen receptors (ER) (Clone 1D5 DAKO Corporation at dilution rate of 1:50); progesterone receptors (PR) (Clone PR 636 DAKO Corporation at 1:50); Her2/neu (CB11; Novocastra Laboratories, Newcastle, U.K. at 1: 50); and Bcl-2 (mouse monoclonal antibody clone 124; Dako at 1:40). The avidin-biotin technique was applied using diaminobenzidin (DAB) for visualization and hematoxylin for counterstaining. Appropriate negative controls, consisting of histological sections of each case processed without the addition of primary antibody, were prepared for each antigen, along with a positive control sections.

Evaluation of immunohistochemistry

Immunohistochemical results were scored semiquantitatively by two pathologists. A cutoff value was applied to each marker to indicate positive or negative staining (Callagy et al., 2006). For hormone receptors, tumors were considered positive if at least 1% of the tumor cells showed unequivocal nuclear staining (Kreike et al., 2007). For Her2/neu, membranous staining was scored for as follows: 0, no staining or faint incomplete staining in <10% cells; 1, faint incomplete staining in >10% cells; 2, weak to moderate complete staining in >10% cells; 3, strong complete staining in >10% cells. Score 3 was considered as positive (Callagy et al., 2006; Kreike et al., 2007). Cytoplasmic staining was scored for Bcl-2. Both the intensity of staining and the percentage of positive cells were recorded and a cutoff value of 10% was used (Callagy et al., 2006). Cases were regarded positive when they showed either moderate or strong staining for these markers (Park et al., 2002).

Selection of triple-negative and non-triple negative tumors

The 77 cases of invasive breast carcinoma were divided into two groups according to the immunostaining results of ER, PR, and Her2/neu: triple negative group (TNBC; n=23); which includes tumors lacking immunohistochemical expression of ER, PR and Her2/neu; and non-triple negative group (non-TNBC; n=54); that included ER and/or PR and/or Her2/neu positive tumors (Kreike et al., 2007).

Statistical analysis

Finally, results of Bcl-2 immunohistochemical expression were correlated with the well accepted prognostic factors in breast cancer including: patient's age, tumor histological subtype, grade, nodal status and tumor size. Pearson chi-square test was used in the statistical analysis of the data and significance was established at $p \leq 0.05$.

Results

This study compares Bcl-2 immunohistochemical expression between triple negative breast carcinoma (TNBC) and non triple negative breast carcinoma (non-TNBC) groups. Table 1 summarizes the correlation between Bcl-2 expression and the clinicopathological variables of the 23 triple negative breast carcinomas (TNBC). Bcl-2 was expressed in 74% of TNBC. Eighty five percent of invasive ductal carcinomas expressed Bcl-2 (Figure.1), while all lobular carcinomas were negative. There was a significant statistical correlation between Bcl-2 expression and histopathologic subtype of breast carcinoma (p=0.002).

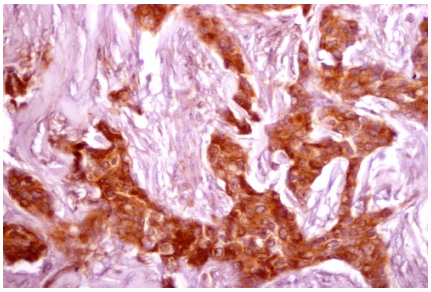

Figure 1. Cytoplasmic Bcl-2 Expression in Invasive Ductal Breast Carcinoma (immunohistochemistry, x 200)

Table 1. Correlation between Bcl-2 Expression and Clinicopathological Variables of the 23 Triple Negative Breast Carcinomas

Clinicopathological variables	Bcl-2 expression				P-value of chi Square test
	Bcl-2 positive		Bcl-2 negative		
	No	%	No	%	
Age :					
< 50years (16 cases)	13	81.30	3	18.70	0.226
≥50years (7 cases)	4	57	3	43	
Histological subtype:					
Invasive ductal (20 cases)	17	85	3	15	0.002*
Invasive lobular (3 cases)	0	0	3	100	
Tumor grade:					
1, 2 (16 cases)	13	81.30	3	18.70	0.226
3 (7 cases)	4	57	3	43	
Nodal status:					
Positive (19 cases)	13	68.40	6	31.60	0.191
Negative (4 cases)	4	100	0	0	
Tumor size:					
<2 cm (4 cases)	4	100	0	0	0.191
≥2 cm (19 cases)	13	68.40	6	31.60	
Total (23 cases)	17 (74)		6 (26)		

*significant p value ≤0.05

Table 2. Correlation between Bcl-2 Expression and Clinicopathological Variables of the 54 Non-triple Negative Breast Carcinomas

Clinicopathological variables	Bcl-2 expression				P-value of chi Square test
	Bcl-2 positive		Bcl-2 negative		
	No	%	No	%	
Age :					
< 50years (22 cases)	19	86.40	3	13.60	0.033*
≥50years (32 cases)	19	59.40	13	40.60	
Histological subtype:					
Invasive ductal (41 cases)	28	68.30	13	31.70	0.553
Invasive lobular (13 cases)	10	77	3	23	
Tumor grade:					
1, 2 (47 cases)	34	72.30	13	27.70	0.411
3 (7 cases)	4	57	3	43	
Nodal status:					
Positive (40 cases)	24	60	16	40	0.005*
Negative (14 cases)	14	100	0	0	
Tumor size:					
<2 cm (4 cases)	4	100	0	0	0.177
≥2 cm (50 cases)	34	68	16	32	
Total (54 cases)	38 (70.4)		16 (29.6)		

*significant p value ≤0.05

Bcl-2 was more frequently expressed in younger patients (81.3%) and in lower grade tumors (81.3%), negative nodal status (100%) and small size tumors (100%); but no significant statistical correlation was detected between the expression of Bcl-2 and these variables in TNBC.

Table 2 summarizes the correlation between Bcl-2 expression and the clinicopathological variables of the 54 non-triple negative breast carcinomas (non-TNBC). Bcl-2 was expressed in 70.4% of non-TNBC. Bcl-2 expression was higher in younger patient's age (86.4%) and in negative nodal status (100%). Significant statistical correlations were detected between Bcl-2 and both variables (p=0.033 and 0.005 respectively).

There was a higher Bcl-2 expression in invasive lobular carcinomas (77%), lower grade tumors (72.3%) and small size tumors (100%), but no significant correlation was found between the expression of Bcl-2 and any of these variables in non-TNBC.

Discussion

One of the major challenges of breast cancer is to define accurate predictive factors that allow the selection of adjuvant therapy which ensures the most benefits and the least harm for the patient. Therefore, various biomarkers are used as a complement to clinicopathological prognostic factors (Munster and Norton, 2001; Callagy et al., 2006; Cecka et al., 2008). Many investigators have focused on gene expression microarray studies and others have used protein expression profiling by immunohistochemistry as a practical alternative for refining classification and prognostication in invasive breast cancer (Munster and Norton, 2001; Callagy et al., 2006).

This article represents one of a few studies which compared Bcl-2 expression between triple negative and non-triple negative invasive breast carcinomas. In this study, immunohistochemistry was used to measure protein expression levels of several biomarkers including Bcl-2, estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (Her2) in 77 cases of breast cancer who were divided into two groups triple-negative (TNBC) and non- triple-negative (non- TNBC). In contrast gene expression signatures, immunohistochemical analysis of Bcl-2 protein expression is a simple, well-validated, inexpensive and widely available test (used routinely in diagnostic pathology of low-grade lymphoproliferative disorders) (Dawson et al., 2010).

In the present work, the pattern of Bcl-2 immunostaining was cytoplasmic, a compatible feature with its localization in the outer membrane of the mitochondria (Park et al., 2002).The frequency of Bcl-2 expression in the current study was slightly higher in TNBC (74%) in contrast to non-TNBC (70%). This is not surprising because a recent study proved that Bcl-2 is a prognostic factor in women with both ER positive and ER negative disease and also in women with both Her2 negative and Her2 positive disease and its positivity is maintained across various molecular subtypes of breast cancer (Dawson et al., 2010). Although the frequency of Bcl-2 expression ranged from 69-73% in previous studies (Dawson et al., 2010; Kallel-Bayoudh et al., 2011), a controversy exists because they found that TNBC tend commonly to have lower Bcl-2 expression compared to non-TNBC (Tawfik et al., 2010; de Ruijter et al., 2011), moreover, Bcl-2 oncogene expression was found to be statistically greater in the ER positive breast tumors compared to ER negative tumors (dos Santos et al., 2008). Such discrepancies between the studies are likely to be due to the wide variation between the methodologies and types of cases studied (Cecka et al., 2008).

Similar to a previous study (Thomadaki et al., 2007), increased expression of Bcl-2 was found in patients belonging to the age groups <50 years (81.3% in TNBC and 86.4% in non-TNBC) but a significant correlation between the expression of Bcl-2 and younger age was found in non-TNBC only. On the other hand, Nadler et al. (2008), found no association between expression of Bcl-2 and patient's age.

To further explore differences in the correlation between Bcl-2 expression and clinicopathological

variables of TNBC and non- TNBC. We found a strong correlation between Bcl-2 expression and ductal carcinomas in TNBC; on the contrary there was a higher Bcl-2 expression among lobular carcinomas in non-TNBC group. The most likely explanation is the predominance of the ductal carcinomas among TNBC than in non-TNBC and the lower proportion of TNBC tumors with either lobular or mixed ductal and lobular features (Tawfik et al., 2010). In this regard, Mottolese et al. (2000), stated that, Bcl-2 expression seems to be of prognostic value only in lobular carcinomas and Mathieu et al. (2004), demonstrated that invasive lobular carcinomas achieve a lower response to therapy than ductal carcinomas because of their Bcl-2 negative immunohistochemical profile .

In this study there was a trend for poorly differentiated (grade 3) tumors to be Bcl-2 negative (43%) in contrast to low grade tumors in both TNBC and non-TNBC. Other studies have also noted that poorly differentiated breast carcinomas lack Bcl-2 reactivity (Bhargava et al., 1994; Park et al., 2002), and others found a significant inverse association with expression of Bcl-2 and increasing tumor grade (Callagy et al., 2006), suggesting that loss of Bcl-2 expression may reflect a loss of differentiation (Bhargava et al., 1994). On the contrary, other studies disclosed no association between expression of Bcl-2 and nuclear grade (Cecka et al., 2008; Nadler et al., 2008).

So far, the strongest predictive and prognostic factor has been the number of affected lymph nodes (Munster and Norton, 2001), yet within the lymph node-positive subset and the lymph node-negative subset of patients there is variability in prognosis (Nadler et al., 2008). In this study, Bcl-2 was more frequently associated with negative nodal status (100%) in both TNBC and non-TNBC and there was a significant inverse correlation with nodal positivity in non-TNBC group. This is consistent with the results obtained previously (Park et al., 2002; Neri et al., 2006), which indicate that the expression of Bcl-2 is a marker of breast cancer with reduced capability of distant colonization even in presence of lymphovascular invasion (Neri et al., 2006). On the contrary, Nadler et al. (2008), found no association between expression of Bcl-2 and nodal status and Dawson et al. (2010), verified the prognostic impact of Bcl-2 positivity regardless of lymph node status.

As expected, all small size tumors expressed Bcl-2 in contrast to about 68% of larger size tumors in both TNBC and non-TNBC, but there were no significant correlation in accordance with a previous study (Cecka et al., 2008). In the same way, Callagy et al. (2006), found an association between Bcl-2 expression and small tumor size. On the contrary other studies, found no association between expression of Bcl-2 and tumor size (Park et al., 2002; Nadler et al., 2008).

In conclusion, the work reported here is one of few studies yet to examine the prognostic role of Bcl-2 in breast cancer in triple negative and non-triple negative breast cancer. In conclusion, Bcl-2 can be combined to the well accepted clinico-pathologic prognostic factors to improve prognostication of invasive breast cancer. The frequency of Bcl-2 expression was identical across both immuno-phenotypes of breast cancer, although different prognostic factors correlated with Bcl-2 expression among triple negative and non- triple negative breast cancers.

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