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Molecular characterization of apoptosis by the immunohistochemical evaluation of Bcl-2 in breast cancer

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Abstract

Purpose: Immunohistochemical markers are used to classify breast cancer into molecular subtypes biologically and behavior distinct. The Bcl-2 gene has been implicated in a number of cancers and it is also thought to be involved in resistance to conventional cancer treatment. The aim of this study was to investigate apoptosis in breast cancer cells and in molecular groups using IHC expression of Bcl-2. *Materials and Methods*: Our study included 61 patients that been followed up five years since diagnosis. The traditional prognostic factors: age, tumor size, histological type, histological grade, clinical stage and the status of the lymph nodes were used for primary morphological evaluation. Molecular classification of cases and Bcl-2 assessment was performed by immunohistochemistry in agreement with data from the literature. *Results*: More than an half of tumors were positive for Bcl-2 showing a favorable response to endocrine therapy. The average age of the patients, hormonal status, tumoral diameter and histological grade showed significant differences statistically. Most of positive cases for Bcl-2 belong to the luminal tumor group, while non-luminal tumors have a negative reaction. When we compared well-known histological types, we noticed insignificant issues regarding score groups for Bcl-2; in exchange, when they were compared into molecular groups, we obtained the strongest statistical correlation. *Conclusions*: Assessment of apoptosis by Bcl-2 leads to the identification of molecular groups with different immunohistochemical and clinical features and different survival rates, better for Bcl-2 positive tumors and worse in Bcl-2 negative tumors.

Keywords: breast cancer, apoptosis, immunohistochemistry.

Introduction

Breast cancer is a clinically, pathologic, therapeutic heterogeneous disease; classically histological classifications do not fully capture the varied clinical course of this disease. Histological type, grade, tumor size, lymph node involvement, and estrogen receptor (ER), progesterone receptor (PR) and HER-2 receptor status all influence prognosis and the probability of response to systemic therapies. Based on the recent DNA microarray studies on breast cancer cases, distinct molecular subtypes of breast carcinoma were identified with different clinical outcomes [1]. Results from reverse transcription polymerase chain reaction and DNA microarrays led to novel risk stratification methods and to new molecular classification of breast cancer. However, large-scale subtyping using gene expression profiling from formalin-fixed, paraffin-embedded samples is not currently feasible. Therefore, immunohistochemical (IHC) markers have been used as surrogates for DNA microarray in subtyping breast cancer.

Apoptosis is the process of programmed cell death that may occur in multicellular organisms. The process of apoptosis is controlled by a diverse range of cell signals, which may originate either extracellular (extrinsic inducers) or intracellular (intrinsic inducers). Extracellular signals may include toxins [2], hormones, growth factors, nitric oxide [3] or cytokines, that must either cross the plasma membrane or transduce to effect a response. These signals may positively (i.e., trigger) or negatively (i.e., repress, inhibit, or dampen) affect apoptosis. Bcl-2 belongs to the Bcl family of proteins that regulate apoptosis; whether a cell undergoes apoptosis or survives depends on the relative expression and dimerization status of the proapoptotic (Bax, Bcl-xs, Bas, Bik/Nbk, Bid, and Bag-1) and antiapoptotic (Bcl-2, Bcl-XL, Bcl-w, A1, and Mcl-1) proteins. The Bcl-2 gene has been implicated in a number of cancers, as well as schizophrenia and autoimmunity. It is also thought to be involved in resistance to conventional cancer treatment. This supports a role for decreased apoptosis in the pathogenesis of cancer. An increase in Bcl-2 shifts the balance in favor of cell survival.

Based on more recent gene expression studies, Carey LA *et al.* [4] updated the IHC subtype definition as luminal A (ER+ and/or PR+, HER2-), luminal B (ER+ and/or PR+, HER2+), HER2+/ER- (ER-, PR-, HER2+), basal-like (ER-, PR-, HER2-, CK5/6+) and unclassified (negative for all five markers). These molecular differences have been shown to correlate with clinical features, such as survival, prognosis and treatment sensitivity.

A number of studies reported a significant positive association between Bcl-2 positivity and increased

overall survival in node-positive breast cancer patients treated with chemotherapy and/or hormone therapy [5, 6], whereas no association between Bcl-2 expression and prognosis could be demonstrated in patients that did not receive adjuvant therapy [6, 7]. Thus, it has been suggested that Bcl-2 expression may be a useful predictor for response to chemotherapy in breast cancer patients.

Materials and Methods

Our study included 61 female patients, aged between 31 and 90 years, who were diagnosed and operated at the Municipal Hospital Timişoara, that were followed up five years since diagnosis. The traditional prognostic factors: age, tumor size, histological type, histological grade, clinical stage and anatomical status of the lymph nodes were use for primary morphological evaluation. Based on these parameters, we set the tumors in the TMN system and the anatomoclinical stages and we calculated the Nottingham prognostic index (NPI), and lymph node prognosis index (LPI). Molecular classification of selected cases was performed by IHC technique in agreement with data from the literature [1, 2, 8]. Proliferation capacity was IHC assessed by Ki-67 index.

Formalin-fixed paraffin-embedded tissue, which had been stored at individual patient, was used in the analysis. For the immunohistochemical investigations have been used sections with thickness of 5 μm and the most technique was EnVision. It is a technique with extremely high sensitivity that can detect antibodies to antigens in very dilute quantities limited (Table 1).

Table 1 – ***Antibodies used for IHC and systems work***

Monoclonal antibody	Source	Clone	Antigen retrieval	Incubation period	Technique	Staining
Anti Bcl-2	Dako	124	40 minutes	30 minutes	EnVision	Cytoplasmic
Anti-ER	Dako	1D5	Microwaves, 40 minutes	30 minutes	EnVision	Nuclear
Anti-PR	Dako	PgR 636	Microwaves, 30 minutes	30 minutes	EnVision	Nuclear
Anti-HER2-neu	Dako	C-erbB2	Microwaves, 40 minutes	30minutes	HercepTest	Membrane
Anti-CK5/6	Dako	D5/16B4	30 minutes	30 minutes	LSAB+	Cytoplasmic
Anti-Ki-67	Dako	MIB-1	20 minutes	10 minutes	EnVision	Nuclear

ER and PR were scored using the Allred scoring system and all cases with 10% positive cells were considered positive. HER2 was scored according to HercepTest criteria 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. All cases with a score of 2+ or 3+ were considered HER2 positive. Interpretation for Ki-67 was as follows: low Ki-67 (below 10% of immunoreactive tumor cells), intermediate index (positive tumor cells between 10–20%) and high index (more than 20% positive tumor cells).

After Callagy GM *et al.* [9] is considered positive for Bcl-2 a limit of 10% positive tumor cells with distinct cytoplasmic pattern. IHC criteria interpretation for Bcl-2 were: negative score 0 -0% and score 1 between 0–10%; positive 10–50% positive cells score 2+; more than 50% positive cells score 3+ (Figures 1 and 2).

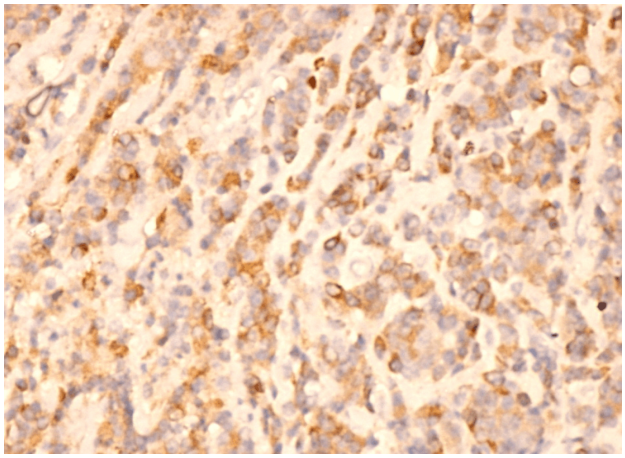

Figure 1 – ***Mixed (ductal and lobular) invasive carcinoma. Moderate positive immunostaining for Bcl-2 (ob. ×40).***

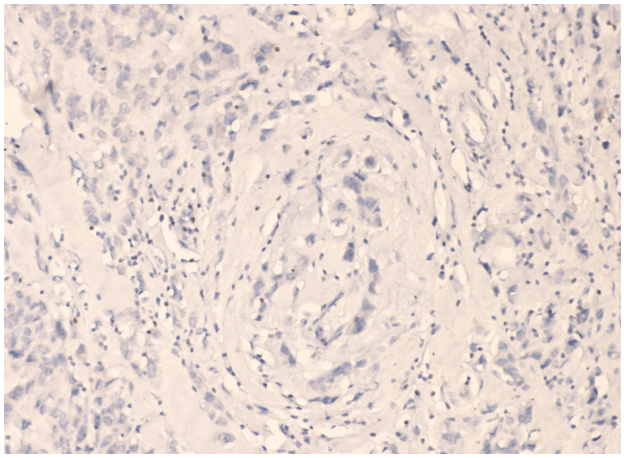

Figure 2 – ***Infiltrating ductal carcinoma. Negative immunostaining for Bcl-2 (×20).***

Whatever system is necessary to use positive and negative external control and/or internal. The most convenient internal positive control is represented by the tumor adjacent normal breast tissue, specifically the positive reaction with nuclear devices in ductal and lobular epithelial cells. Interpreting the results, positive control tissues were examined first followed by the negative control sections to check non-specific staining followed by examination of the evidence.

Statistical analysis (frequencies and percentages for qualitative variables, statistical comparison – chi-square) of the results was performed by known criteria:

$p<0.05$ significant differences. Kaplan–Meier method was used to assess survival.

Results

The group evaluated a rate of 54.1% of tumors were positive for Bcl-2; in cases with 3+ reaction for Bcl-2 all were positive for ER, with an identical value for PR, showing a favorable response to endocrine therapy. Cases with negative reaction show an average age of 53 years, while for cases with positive expression was 62 years. Among premenopausal cases (16 cases), mostly have a negative reaction for Bcl-2 (score 1+ and 0) and postmenopausal cases, most of them are the Bcl-2 positive (score 2+ and 3+) (Table 2).

Table 2 – ***Bcl-2 expression according to clinical-pathological parameters***

Parameters		Bcl-2 negative tumors		Bcl-2 positive tumors	
		Score 0	*Score 1+*	*Score 2+*	*Score 3+*
No. of cases	*61*	19	9	17	16
Age [years] (p=0.001)	*Average*	52.6	53.9	61.1	64.2
Hormonal status (p=0.001)	*Premenopausal*	9	4	2	1
	Menopausal	10	5	15	15
Tumoral diameter [cm] (p=0.04)	*Average*	4.7	4.7	4	2.4
	<2	1	1	4	4
	2–5	11	3	8	11
	>5	7	5	5	1
Histopatological type (p=0.1)	*Ductal*	14	5	12	11
	Lobulary	1	0	1	2
	Medullary	1	3	0	0
	Mixed	3	1	4	4
Histological grade (p=0.048)	*G1*	0	0	1	3
	G2	14	6	11	13
	G3	5	3	5	0
Lymph node metastases (p=0.62)	*Present*	13	6	15	9
	Absent	5	2	2	9
Anatomo-clinical stage (p=0.08)	*I*	0	0	0	1
	II	10	5	8	15
	III	8	4	6	0
	IV	1	1	2	0

We found that the percentage of Bcl-2 positive cases, the percentage scoring 3+, decreases with increasing tumor grade so that the G3 tumors who were not present positive 3+. All well differentiated infiltrating ductal carcinomas (G1) show the Bcl-2 positive and score 3+ was found in 75% of the cases. Moderately differentiated tumors (44 cases) had a rate of 54.5% Bcl-2 positive cases, remaining cases being negative, but the difference is small. G3 poorly differentiated carcinomas presented positive reaction for Bcl-2 only 38,4% of cases, all scoring 2+, but these differences had a borderline statistical significance (p=0.048).

Average tumor diameter was more than 4.7 cm in patients with absence of Bcl-2 expression, while positive tumors had a tumor diameter less than 3.2 cm. An average diameter less than 2 cm were in 20% of cases Bcl-2 negative, the remainder being positive and among these 60% had 3+ score. In contrast, tumors over 5 cm in diameter were characterized by the absence of Bcl-2 expression in 66.7% of cases, while those with Bcl-2 expression were 33.2% percentage of which two thirds were 2+ score.

Invasive ductal carcinomas were either negative (45.2%) either positive (54.8%) for Bcl-2, most showing score 0. Invasive lobular carcinomas were positive rate of 66.6% and only one case was negative. Medullary carcinomas were all negative, ductal carcinoma and lobular mixed types were positive in a proportion of 66.7% (Figures 3 and 4). Among cases without lymph node metastases, most of them are Bcl-2 positive with a rate of 61.1%, of which over 50% were 3+ score. Among cases with lymph node metastases, 55.8% are positive for Bcl-2.

Anatomoclinical stage although was not statistically significant, stage I was only Bcl-2 positive score 3+, followed by stage II at the rate of 39.5%, most cases been 3+. In stage III, 44.4% of tumors were negative for Bcl-2 score 0 and stage IV was equally distributed between positive and negative cases. Tumors having a favorable prognostic index Nottingham were in 41.7% positive cases with score 3+, which value decreases to 28% for tumors with moderate index and is reduced in tumors with poor prognostic index to 16.7%. Tumors with a negative reaction for Bcl-2 showed a favorable prognostic index Nottingham in 16.7% of cases, a moderate index in 32% of cases and unfavorable in 16.7% of index cases. Lymph-node prognosis index showed the same variations, like those described in Nottingham prognostic index, the percentage of cases with poor prognosis decreases with increasing Bcl-2 score, while the percentage of cases with good prognosis increases with increasing evaluation score.

High levels of Bcl-2 expression are strongly associated with positive expression of receptors for estrogen and progesterone and the response to hormonal

therapy. Bcl-2+ evaluated cases show in 100% positive reaction to estrogen receptors and in 93.6% of cases positive reaction to progesterone receptors. 52.7% of ER+ cases presented the highest percentage of immunoreactive cells and these are cases with good response rate to hormonal therapy and have the lowest recurrence rate.

Assessing the Bcl-2 expression according to molecular classification, we observed that luminal A type tumors expressed Bcl-2 marker at a rate of 92.3%, the luminal B in 60% of cases, while the remaining molecular subtypes showed no expression (Figure 5).

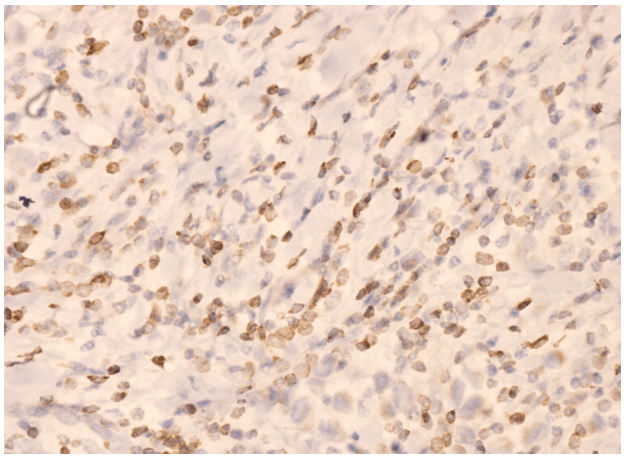

Figure 3 – ***Infiltrating lobulary carcinoma. Moderate positive immunostaining for Bcl-2 (ob. ×40).***

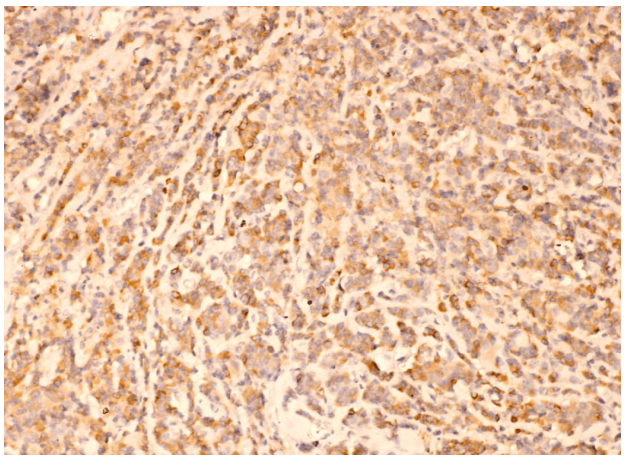

Figure 4 – ***Lobulary/ductal infiltrating carcinoma. Moderate positive immunostaining for Bcl-2 (ob. ×20).***

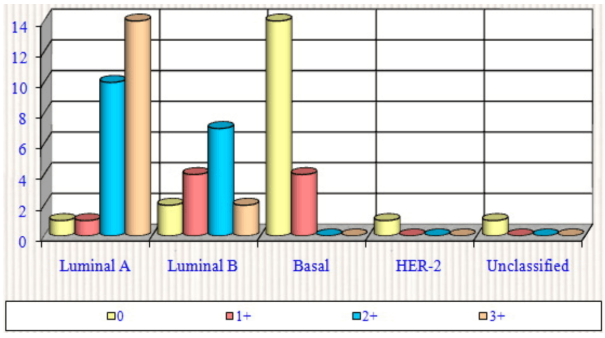

Figure 5 – ***Molecular type of tumors.***

Most of positive for Bcl-2 cases are luminal tumor group and between them dominate the luminal subtype A. Among non-luminal tumors, the basal tumors has a negative reaction score 0 in 77.8% cases, the rest being 1+. HER2-type tumors and unclassified were classified the score 0 (Table 3).

Table 3 – ***Bcl-2 expression according to molecular subtype and survival***

Parameters		Bcl-2 negative tumors		Bcl-2 positive tumors	
		Score 0	*Score 1+*	*Score 2+*	*Score 3+*
Molecular subtype (p=0.0001)	*Luminal A*	1	1	10	14
	Luminal B	2	4	7	2
	Basal-like	14	4	0	0
	HER-2	1	0	0	0
	Unclassified	1	0	0	0
Nottingham prognostic index (p=0.44)	*NPP*	9	6	5	4
	NPM	8	3	7	7
	NPG	2	2	3	5
Lymph node prognosis index (p=0.38)	*LPP*	5	0	3	0
	LPM	10	5	8	8
	LPG	4	4	6	8
Survival rate at 5 years		42.1%	66.7%	88.2%	100%

A high level of Bcl-2 expression is strongly associated with positive estrogen and progesterone receptors and response to hormonal therapy, respectively. The evaluated Bcl-2 positive cases, present in 100% of cases and positive estrogen receptors as the receptors for progesterone in 93.6% of cases. In addition, 52.7% of ER positive cases have positive 7/8 Allred score, these cases representing a good response rate to hormone therapy. Therefore, cases that are Bcl-2+, but which are ER positive and PR positive are half the cases considered the best answer to hormone therapy and have the lowest rate of recurrence.

Phenotype Bcl-2+/ER+/PR+ (33 cases) associated the lack of HER2 expression in 24 cases, the rest being positive. Proliferation capacity of these tumors was especially low, 22 with low Ki-67, six intermediate and high only five cases. These tumors were CK5/6 positive only in five cases. All this indicates an immunophenotype with a favorable prognosis, which is demonstrated by the prognostic indicators, used in evaluation and the relative survival rates in five years (93.5%).

Phenotype Bcl-2-/ER-/PR- (19 cases) was more aggressive. Proliferation capacity assessed by Ki-67 index was tall in 15 cases, intermediate in two and low throughout the two. Most of these (18 cases) were HER2 negative and all CK5/6 positive. Unfavorable prognosis is supported by evidence a Nottingham and lymphonodal index that were unfavorable. The 5-year survival rates were 63.1%.

Data from the literature on the relationship between Bcl-2 and proliferative activity are divergent. There are authors who have shown that the expression of Bcl-2 is significantly more frequent in breast carcinoma with Ki-67 index decreased, according to others there was no association between Bcl-2 and Ki-67 status. Proliferation capacity was assessed by Ki-67 index seeing that in highly proliferative tumors negative Bcl-2 were the

most while lower proliferation showed some Bcl-2 positive tumors (p=0.00049). Regarding to the Bcl-2 positive tumors, 66.6% (22 cases) had a low Ki-67, 18.8% (six cases), intermediate type and only 15.1% (five cases) tall. Bcl-2 negative tumors were highly proliferative especially: Ki-67 was tall in 51.7% (15 cases), intermediate in 27.5% (eight cases) and low in 17.2% (five cases).

Evaluation of patient survival curve showed significant differences according to the expression of Bcl-2. Patients whose tumors did not express Bcl-2, score 0 had a median survival rate of 42.1%, average survival was 53 months and those with carcinomas that were characterized by a 1+ score had a lower survival rate of 66.7%, the average survival being 55 months. Patients whose tumors expressed Bcl-2 score 2+ showed an average survival rate of 88.2%, the average survival being 96 months and tumors with the highest expression 3+ have the highest survival rate of 100%, with an average survival of over 96 months (Figure 6).

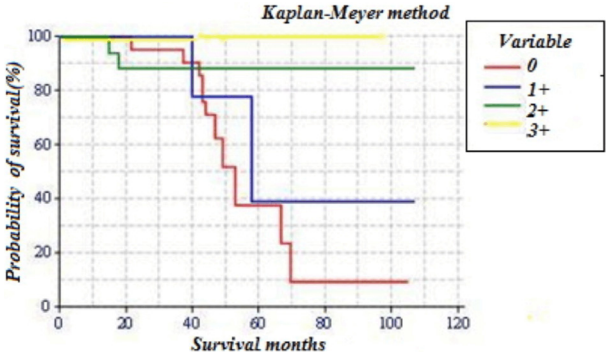

Figure 6 – ***Survival curves according to the evaluation score for Bcl-2.***

Discussion

Whether the prognostic role of Bcl-2 is consequent on its role in apoptosis or whether proposed non-apoptotic functions of Bcl-2 are somehow involved is unknown. Non-apoptotic functions have been described; interestingly, in vitro experiments have revealed that high levels of Bcl-2 can result in dramatic growth inhibition in different cell types. Indeed, a role in prolonging the cell cycle has been proposed [10–12].

In breast cancer, Bcl-2 expression is associated with markers of better differentiation (e.g., grade 1 lesions, which are ER positive with low proliferative status) as we confirmed in this work. Most previous studies of Bcl-2 in breast cancer have also shown a favorable association between Bcl-2 positivity and outcome at least in univariate analysis. In the breast, Bcl-2 is expressed in normal glandular epithelium and is up-regulated by estrogen possibly as a result of direct transcriptional induction with negative regulation by p53-dependent mechanisms [13–15]. In breast cancer has reported a strong correlation between the presence of Bcl-2 and estrogen receptor (ER) positivity; no relationship was found with lymph node status, tumour size or differentiation type and an inverse relationship with immunostaining of c-erbB2 protein. Similar findings were reported in a series of 283 breast cancers from lymph node negative patients; the highest fraction of Bcl-2 positive cells was found in small, ER-positive, slowly proliferating and p53-negative tumors and a stronger association was seen between Bcl-2 and p53 than between these variables and proliferative activity [16].

Conclusions

The evaluation group of 61 tumors, we obtained a percentage of positive cases for Bcl-2 of 54% and a quarter of all patients, are cases with score 3+. It is noted that there are significant differences (p<0.001) regarding the hormonal status, cases which from premenopausals were more frequently negative and the postmenopausal ones were positive. In the group evaluated, Bcl-2 expression correlates inversely with the degree of histological differentiation as that occurs with increasing proportion of cases decreased Bcl-2+. Small tumors were Bcl-2 positive while larger tumors were negative. Most tumors without lymph node metastases are Bcl-2 positive (61.1%), while 38.9% of cases with lymph node metastases were negative for Bcl-2.

Evaluation of Bcl-2 expression, taking into account the molecular classification, showed that positive tumors represent 92.3% of all luminal A tumors and 60% of luminal B. Instead non-luminal tumors (basal-like, unclassified and HER2) are negative.

There are statistically insignificant differences regarding Nottingham or lymph node index groups. It is noted that the poor prognosis group the percentage Bcl-2 positive cases decreases with increasing score, while inside favorable prognostic group increasing number of cases with the increased assessment score. Confirming this, five years survival rates were significantly better for Bcl-2 positive patients and worse in patients Bcl-2 negative.

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