RESEARCH STUDIES

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B-cell lymphoma-2 receptor in human primary breast and its lymph node metastases: more than a surrogate marker

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Abstract

Background: Due to its anti-apoptotic and anti-proliferative contradictory functions, BCL2 role in breast carcinoma progression is not clearly understood. The purpose of this study was to highlight BCL2 expression during metastatic progression of invasive breast carcinoma of no special type (NST).
Materials and methods: The specimens, primary tumors and corresponding lymph node metastases (LNM) from 84 patients were immunostained for estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor (HER)-2, basal cytokeratin CK5, nuclear protein Ki67 and B-cell lymphoma (Bcl)-2 receptor.
Results: BCL2 expression was higher at primary site than in axillary metastases. Its score correlates positively with hormone receptors' level and negatively with HER2, CK5 and Ki67 at both sites. Switch of molecular profile was determined in 22.62% of cases. BCL2 expression was not influenced by subtypes switch. Changes of BCL2 expression were found in 25% of cases with stable molecular subtype. The Luminal A and Luminal B/Ki67 were encountered in the majority of BCL2 transitions, mainly from positive to negative state.
Conclusions: Molecular subtypes and BCL2 expression are not stable during tumor progression and metastatic development. In the present study we established immunohistochemically that BCL2 is not influenced by subtypes' transitions. BCL2 switches were encountered only in cases with a stable HER2, Luminal A or B phenotypes. We expect a further confirmation of our results by other research groups.
Key words: BCL2, breast carcinoma, immunohistochemistry, molecular subtypes, metastases.

Introduction

Breast carcinoma is the most common cause of death among women, and despite all efforts the drivers of this malignancy are not completely elucidated. Cancer occurs as the result of a disturbance in the balance between cell growth and cell death. Over-expression of anti-apoptotic genes or under-expression of pro-apoptotic genes can result in the lack of cell death, mechanisms that have been demonstrated in breast cancer too. Besides well-known prognostic factors, such as tumor size, histological type and grade, vascular invasion, identifying of new molecular factors has become the objective of many research studies. One of these potential markers is B-cell lymphoma (Bcl)-2 receptor.

BCL2 is a member of regulator proteins that regulate cell death, by either acting as pro-apoptotic or anti-apoptotic [1, 2]. BCL2 is specifically considered as an important anti-apoptotic protein and is thus classified as an oncogene. This protein, apart from its well-known inhibition of apoptosis, can also inhibit progression of the cell cycle by delaying entry into the S phase and maintaining cells in the G0 phase.

BCL2 in addition to HER2 and p53 is considered a tumor-related protein that has the potential to further improve individualization of patient management, by predicting response to chemotherapy, hormone therapy and radiotherapy [3]. Due to its anti-apoptotic function BCL2 is considered an important factor in the modulation of hormonal/anti-hormonal responsiveness exhibited by tumors [4]. Patients with elevated BCL2 immunostaining appeared to have the greatest benefit from endocrine therapy. In addition, BCL2 overexpression is associated with favorable outcome and its effect in relationship to estrogen receptor (ER) status. Some data suggest that BCL2 expression is a predictive factor for response to chemotherapy particularly in anthracycline-based chemotherapy [5]. Other report showed this marker has no significant value for the therapeutic strategy [6]. Contrary, clinicopathological data suggests that BCL2 expression correlates with aggressive, prometastatic behavior in breast cancer [7].

In the last decade, the gene analysis led to identification of molecular subtypes and defined the gene-expression prognostic signatures [8, 9]. By analysis of protein expression using immunohistochemistry it has been identified that molecular subtypes characterized with surrogate markers are similar to those derived from gene expression arrays [10, 11]. Nowadays, it seems to be important to investigate the molecular profile of metastases too. Recent data reveal the instability of receptors throughout the metastatic process [12, 13]. This supports the hypothesis that the malignant phenotype is not pre-determined, but continues to evolve throughout its natural history. In comparison to basic, five

commonly accepted markers in breast cancer stratification (ER, PR, HER2, CK5, Ki67), nowadays is not clear the future of BCL2 positive tumor cells in the lymphonodal environment. The aim of the current study was to compare BCL2 expression from primary tumor with corresponding lymph node metastases (LNM) in association with hormone receptors (estrogen (ER), progesterone (PR)), HER2 (human epidermal growth factor receptor-2), basal cytokeratin CK5 status and molecular subtypes.

Material and methods

Patients. In this retrospective study there were analyzed specimens (breast carcinoma of no special type and corresponding axillary lymph node metastases) from 84 patients of 33-86 years old. In all cases patients underwent radical mastectomy and lymph nodes dissection, without prior chemo- and radiotherapy. Histopathological diagnosis was assessed by two pathologists and cases suitable for immunohistochemistry were carefully selected.

Ethical issues. This study was based on patients' informed consent and approved by the Ethics Committee of the Nicolae Testemitsanu State University of Medicine and Pharmacy from Chisinau, the Republic of Moldova (approval number 21/13/31.03.2014).

Specimen processing and immunohistochemistry. The specimens were fixed in 10% phosphate buffered formalin for 24-48h and paraffin embedded (Paraplast High Melt, Leica Biosystems). Primary tumor and its LNM were placed in one block and 5-µm thick step sections were cut. The immunohistochemical assessment included 6 surrogate markers (from Leica Biosystems, except HER2), for ER (clone ER/6F11), PR (clone Pr16), human epidermal growth factor receptor 2 (HER2/polyclonal/ DakoCytomation), marker of proliferation Ki67 (clone K2), basal cytokeratin CK5 (clone XM26) and BCL2 (clone BCL2/100/D5). Incubation with primary antibodies was followed by the use of HercepTest PharmDx Kit (DakoCytomation) and Bond Polymer Refine Detection System (Leica Biosystems, Newcastle Upon Tyne, UK). All cases were evaluated also by FISH as international rules recommend (PathVysion HER-2 DNA Probe Kit II, Abbot). Slides were processed automatically on Leica Bond-Max autostainer (Leica Microsystems GmbH, Wetzlar, Ger-

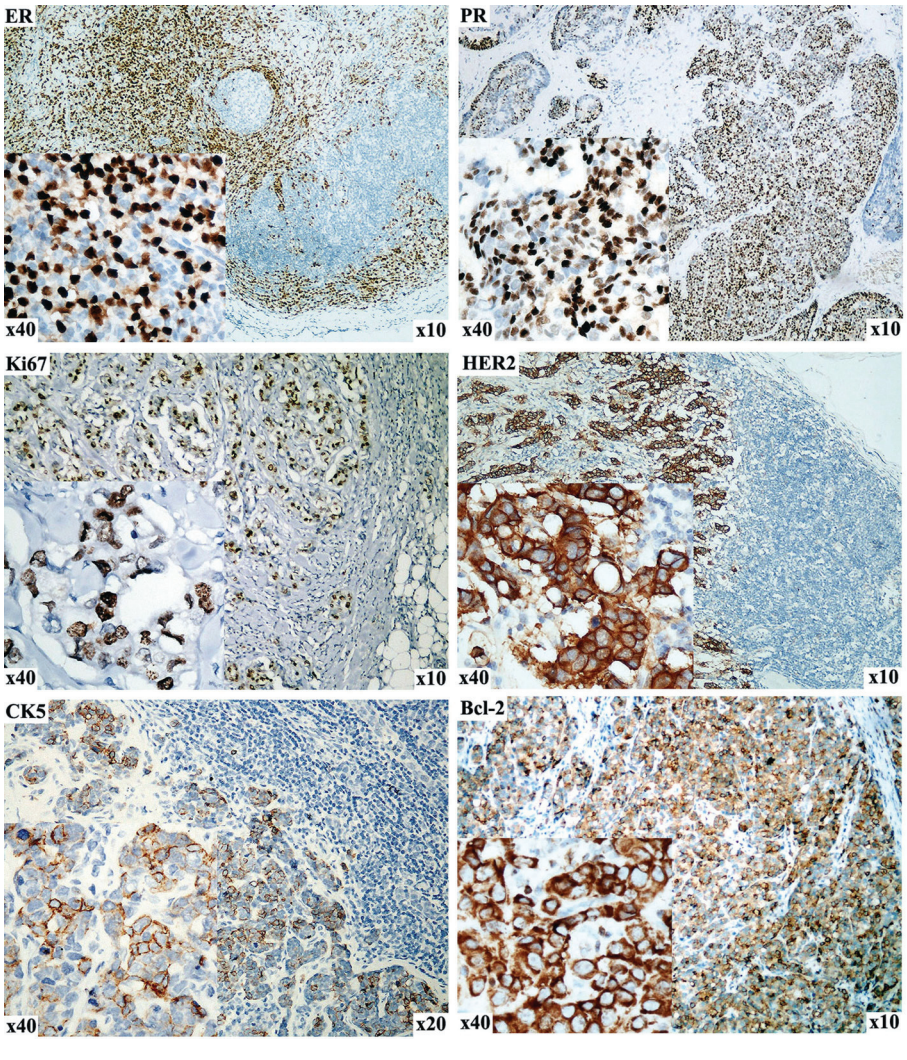

Fig. 1. Representative images of ER, PR, HER2, CK5, Ki67, BCL2 immunohistochemical staining in primary breast carcinoma and corresponding lymphonodal metastases (color version look on the front page of the cover).

many). The hematoxylin solution, Harris modified (HHS32, SigmaAldrich) was used for counterstaining (fig.1).

Microscopic evaluation. The hormone receptors were scored as the percentage of nuclear positively stained cells from at least 1000 cells assessed. We followed the guidelines of ER and PR assessment purposed by Allred et al. [14]. Cases scored +1 – +3 were considered positive. The threshold of positivity was 10%.

The HER2 assessments were done on LeicaBond Oracle HER2 IHC System (LeicaBiosystem). The HER2 status was interpreted in accordance with American Society of Clinical Oncology recommendations [15]. Cases interpreted as +2 and +3 were considered positive. Leica HER2 control slides ensured the control and accuracy of our decisions.

We used a 14% threshold for Ki67. This marker, as well as hormone receptors were counted using a semi-quantitative method performed by Suciu et al. [16].

The basal cytokeratin CK5 was interpreted in Azoulay's et al. manner [17]. Cases evaluated as +1 – +3 were considered positive.

The BCL2 evaluation was based on Callagy et al. recommendations: 0 – no staining; +1 – until 10% of cells show cytoplasmic pattern; +2 – 10-50%; +3 – more than 50% [18]. Cases scored as +2 and +3 were considered positive.

Based on Goldhirsch et al. recommendation we clustered molecular subtypes, as follows: ER^+ and/or PR^+, $HER2^-$, $CK5^-$, Ki67<14% as Luminal A; ER^+ and/or PR^+, $HER2^+$, $CK5^-$ as Luminal B/HER2; ER^+ and/or PR^+, $HER2^-$, $CK5^-$, Ki67>14% as Luminal B/Ki67; ER^+ and/or PR^+, $HER2^+$, $CK5^-$, Ki67>14% as Luminal B/HER2/Ki67; ER^-, PR^-, $HER2^+$, $CK5^-$ as HER2-overexpressed; ER^-, PR^-, $HER2^-$ and $CK5^+$ as Basal-like; ER^-, PR^-, $HER2^-$ and $CK5^-$ as unclassified [19].

Image acquisition and statistical analysis. A Nikon Eclipse 80i microscope with Nikon DS-Fi1 installed camera and Nis-elements BR 2.30 imaging software were used for microscopic evaluation (Nikon Instruments Europe BV). For descriptive statistics and Pearson's correlation assessment WinStat 2012.1 software was used (R. Fitch Software, Bad Krozingen, Germany). For all the tests a value of p£0.05 was considered significant. In order to determine the shifting direction of subtypes (from basal to Luminal and *vice versa*) unclassified subtype was assigned as "1", Basal-like as "2", $HER2^+$ as "3, Luminal B/HER2-"4", Luminal B/HER2/Ki67 –"5", Luminal B/Ki67 –"6" and Luminal A was equated with "7".

Results

Histological assessment revealed that the most frequent histological grade is G2, determined in 45 cases (53.6%), the share of G1 constitutes 6%/5 cases and G3 respectively in 40.5%/34 cases. In relation to BCL2, the majority of positive scores were determined in cases with G2 (36 cases/42.9%) and G3 grades (23 cases/27.4%) In primary tumor the surrogate markers for hormone receptors had the highest rate. BCL2 marker was positive in 62 cases/73.8% (tab. 1).

Table 1

Surrogate markers positivity in primary tu

Score	ER		PR		HER2		CK5		BCL2	
	No	%	No	%	No	%	No	%	No	%
0	16	**19.0**	26	**31.0**	62	**73.8**	73	**86.9**	17	**20.2**
1	3	**3.6**	5	**6.0**	4	**4.8**	5	**6.0**	5	**6.0**
2	9	**10.7**	13	**15.5**	5	**6.0**	4	**4.8**	9	**10.7**
3	56	**66.7**	40	**47.6**	13	**15.5**	2	**2.4**	53	**63.1**
	84 cases/**100%**									

In relation to hormonal receptors the positive BCL2 highest score was determined in cases with high expression of ER and PR receptors (tab. 2). And *vice versa* increasing of the HER2 and CK5 scores leads to decreasing of BCL2 score.

The proliferation marker Ki67 was considered positive (≥14) in 50 cases/59.52% of primary tumors. A positive BCL2 was accompanied with positive Ki67 (≥14) in 32 cases/38.10% and in 30 cases/35.71% with a Ki67<14. In 18 cases/21.43% with high Ki67 level, BCL2 was considered negative.

The most frequent subtype at primary site was Luminal B (45 cases/53.6%), followed by Luminal A (26 cases/31%) and hormone-negative group (13 cases/15.5%). By comparing the molecular profile and BCL2 score the highest rate of positivity was determined in Luminal B (39.29%) and Luminal A subtype (28.57%). The ratio of positive/negative BCL2 marker in HER2 overexpressing subtype was 50/50 – (4.76% positive)/(4.76% negative). Appropriate ratio was determined for Luminal B/HER2/Ki67 – 4.76% positive/3.57% negative.

By comparing molecular profile of primary tumor and its LNM we determined a transition of subtype to another one in 19 cases/22.62%. We have to mention that BCL2 expression was stable in spite of these switches. Transitions of BCL2 score (21 cases/25%) we found in the group, where molecular subtype at both sites is similar (tab. 3).

The highest percentage of BCL2 transition was encountered in Luminal A (8 cases/9.52%) and Luminal B/Ki67 (8 cases/9.52%). HER2-positive cases (4 cases/4.76%) associated in primary tumor with positive BCL2, preserved molecular subtype in metastases, but became BCL2 negative. In the same manner, from positive BCL2 to negative, proceed Luminal B/Ki67 and Luminal A, in 7 cases/8.33% each. The main intention of BCL2 expression throughout metastatic process was rather loss than acquiring – in 19 cases (from 21 changed), direction was from positive in primary tumor to negative in lymph node metastases. This is visible and by comparing the number of BCL2 positive cases at both sites – 62 cases/73.81% in primary tumor and 44 cases/52.38% in LNM.

BCL2 expression was analyzed in relation to proliferation activity. Tumors were grouped in 2 categories in accordance with Ki67 level: low proliferating (<14) and high proliferating (≥14). In 7 cases of Luminal A, where BCL2 changed during progression from negative to positive, the Ki67 had a low level

Table 2

BCL2 expression in relation to ER, PR, HER2, CK5 surrogate markers score at primary tumor site

BCL2 score	No	%	No	%	No	%	No	%	Total	
	ER score									
	0		1		2		3		No	%
0	10	11.9			3	3.6	4	4.8	17	20.2
1					1	1.2	4	4.8	5	6
2	2	2.4	1	1.2	1	1.2	5	6.0	9	10.7
3	4	4.8	2	2.4	4	4.8	43	51.2	53	63.1
Total	16	19.05	3	3.57	9	10.71	56	66.67	84	100
PR score										
BCL2 score	No	%	No	%	No	%	No	%	No	%
	0		1		2		3			
0	10	11.9	3	3.6	2	2.4	2	2.4	17	20.2
1					2	2.4	3	3.6	5	6
2	3	3.6			2	2.4	4	4.8	9	10.7
3	13	15.5	2	2.4	7	8.3	31	36.9	53	63.1
Total	26	31.0	5	6.0	13	15.5	40	47.6	84	100
HER2 score										
BCL2 score	No	%	No	%	No	%	No	%	No	%
	0		1		2		3			
0	9	10.7			1	1.2	7	8.3	17	20.2
1	4	4.8	1	1.2					5	6
2	7	8.3					2	2.4	9	10.7
3	42	50.0	3	3.6	4	4.8	4	4.8	53	63.1
Total	62	73.8	4	4.8	5	6.0	13	15.5	84	100
CK5 score										
BCL2 score	No	%	No	%	No	%	No	%	No	%
	0		1		2		3			
0	10	11.9	4	4.8	1	1.2	2	2.4	17	20.24
1	5	6.0							5	6
2	8	9.5	1	1.2					9	10.7
3	50	59.5			3	3.6			53	63.1
Total	73	86.9	5	6.0	4	4.8	2	2.4	84	100

(<14). Other 7 cases of Luminal B/Ki67, where BCL2 from positive changed to negative, Ki67 preserved a high rate at both sites. If in Luminal subtypes the proliferation marker preserves similar quotes at both sites, in HER2-overexpressed it changed randomly. In the last 7 cases some correlation of Ki67 level with BCL2 switch was not observed.

The statistical assays revealed significant, positive correlation of BCL2 level with type of molecular profile, level of ER, PR markers and negative correlation with HER2, CK5 and Ki67.

Discussion

Breast carcinoma is the most common cause of death among women. Despite implementation of screening programs the incidence of this tumor worldwide is still increasing. Besides well-known prognostic factors, such as tumor size, histological type and grade, vascular invasion, identifying of new molecular factors became the objective of many research studies. Failure to undergo apoptosis is considered a major mechanism of cancerogenesis and chemoresistance.

A marker which is involved in inhibition of apoptosis is B-cell lymphoma (Bcl)-2 receptor. It has been shown to contribute to oncogenesis because it can transform and immortalize cells in cooperation with c-myc, ras or viral genes. Del Bufalo et al. consider that BCL2 overexpression enhances both tumorigenicity and metastatic potential of tumor cells by inducing metastasis-associated properties [20]. Rochaix et al. found that BCL2 expression in tumors

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Table 3

Molecular subtypes and BCL2 evolution in tumor progression: a comparative study of primary tumor and corresponding metastases

Molecular subtype		BCL2 expression		No. cases	%	
Tm	Mt	Tm	Mt			
Unclassified	Unclassified	-	-	2	2.38	
Unclassified	Unclassified	+	+	1	1.19	
Basal-like	Basal-like	-	-	1	1.19	
HER2	HER2	-	-	3	3.57	
HER2	HER2	+	-	**4**	**4.76**	
Luminal A	Luminal A	-	-	1	1.19	
Luminal A	Luminal A	-	+	**1**	**1.19**	
Luminal A	Luminal A	+	-	**7**	**8.33**	
Luminal A	Luminal A	+	+	13	15.48	
Luminal B/HER2	Luminal B/HER2	-	-	1	1.19	**77.4**
Luminal B/HER2	Luminal B/HER2	+	-	**1**	**1.19**	
Luminal B/HER2/Ki67	Luminal B/HER2	-	-	1	1.19	
Luminal B/HER2/Ki67	Luminal B/HER2/Ki67	-	-	2	2.38	
Luminal B/HER2/Ki67	Luminal B/HER2/Ki67	+	+	1	1.19	
Luminal B/HER2/Ki67	Luminal B/Ki67	+	+	1	1.19	
Luminal B/Ki67	Luminal B/Ki67	-	-	1	1.19	
Luminal B/Ki67	Luminal B/Ki67	-	+	1	1.19	
Luminal B/Ki67	Luminal B/Ki67	+	-	**7**	**8.33**	
Luminal B/Ki67	Luminal B/Ki67	+	+	16	19.05	
Basal-like	Unclassified	-	-	1	1.19	
HER2	Luminal B/HER2/Ki67	-	-	1	1.19	
Luminal A	Unclassified	+	+	1	1.19	
Luminal A	Luminal B/HER2	+	+	1	1.19	
Luminal A	Luminal B/Ki67	+	+	2	2.38	
Luminal B/HER2	Luminal A	+	+	1	1.19	**22.6**
Luminal B/HER2/Ki67	HER2	-	-	1	1.19	
Luminal B/HER2/Ki67	Luminal A	+	+	1	1.19	
Luminal B/Ki67	Basal-like	-	-	1	1.19	
Luminal B/Ki67	Luminal A	-	-	5	5.95	
Luminal B/Ki67	Luminal A	+	+	4	4.76	

Note: Molecular subtypes shifted cases are selected with **Bold**. BCl2 transitions (No and %) are primed with gray color**. Tm –** primary tumor, **Mt –** metastases.

was associated with a better differentiation of the cancers and particularly G1 – 100% of BCL2-positive tumors, G2 – 81%, G3 – 60% [21]. This assumption is in line with our results where majority (70.2%) of BCL2 positive cases was evaluated with G2 and G3 grades. Contradictory, Binder et al. presented a significant inverse correlation between histological grading and immunoreactivity for BCL2, confirmed by Ermiah et al. too [22, 23]. In our assays a statistically significant correlation between histological grade and BCL2 was not found.

Ermiah et al. consider that patients with positive expression of BCL2 had lower recurrence rate than BCL2-negative patients and better survival after median follow-up of 47 months [23]. Recently, Yang et al. found significant relation between BCL2 negativity and good prognosis [5]. All related uniformities could have several explanations: unknown, other than anti-apoptotic function of BCL2, lack of homogeneity in studied group, differences in immunohistochemical assays and the cut-off used to define BCL2 positivity [24]. Lee et al. considered BCL2 positive case as ">0%" of stained cells and Dumontet et al. used a threshold of ">70%" positive cells [25, 26].

BCL2 is considered as modulator of hormonal/anti-

hormonal responsiveness exhibited by tumors. Binder et al. supposed that loss of BCL2 expression seems to induce the loss of hormonal regulation, increased dedifferentiation and deregulated proliferation [22]. Gee *et al.* determined that immunostaining for hormone receptors was strongly associated with that for the BCL2 protein, results which were confirmed by other researches later too [4, 27]. Authors supposed that this protein, like progesterone receptor, is under estrogen regulation via estrogen receptor. These results were confirmed also by a highly significant relationship between response to endocrine therapy and the presence of BCL2 protein. Indeed, BCL2 was more accurate predictor of response than estrogen receptor status. Patients who had a combination of BCL2 high score and elevated ER level received the greatest benefit from endocrine therapy. Our results concerning BCL2 and hormone receptors relationship are similar to Gee et al. [4].

In the literature the BCL2 was inversely related to c-erbB-2 oncoprotein (as well to epidermal growth factor receptor or EGFR) [4, 22]. Petry et al. support the concept that ERBB2 influences the expression of BCL2 family members to induce an anti-apoptotic phenotype [28]. Authors indicate that ERBB2 alters the expression of BCL2 in a way that leads to adverse prognosis. In our assays BCL2 correlated negatively with HER2 expression.

The anti-apoptotic function of BCL2 should predispose tumor to high proliferation. No associations were observed with Ki-67 proliferative status by some researches [4]. More, high proliferative activity assessed by Ki-67 correlated inversely with BCL2 expression in primary tumor in Binde et al. and Zaha et al. experiments [22, 29]. Our results are complementary to above mentioned.

CK5/6-positive breast carcinomas have a low BCL2 expression and high proliferation rate [30]. Same data arise from our study too.

Korsching et al. consider that different cellular subgroups in the female breast give rise to subgroups of breast carcinomas with different protein expression and cytogenetic alteration patterns that may be related to clinical behavior [30]. Approximately 80% of patients present hormone positive tumors. Dawson et al. established that prognostic value of BCL2 was present across molecular subtypes (ER+/Luminal, HER2+, HER2– and triple negative), and was independent of tumor size, grade and stage [31]. Cases with $ER^+/BCL2^-$ pattern had a worse prognosis than those with $ER^-/BCL2^+$.

There are several evidences which affirm the instability of molecular subtypes during metastatic process [12, 13, 32]. In our study BCL2 expression was stable throughout this transition and unexpectedly changed in the group where molecular subtype was the same at both sites. Our results are in line with Subhawong *et al.*, which reported a downregulation of BCL2 expression in metastases in Luminal A subtype [33]. But in this report, patients (17 cases) underwent many cycles of adjuvant therapy prior to study and developed resistance to hormonal therapy. We couldn't find other results concerning relationship of BCL2 expression and molecular subtypes throughout progression.

Regarding the involvement of BCL2 in metastatic process our results are in contradiction with data of other groups, which reported an increase of BCL2 expression in axillary metastases [34, 35]. Another study reported a similar BCL2 expression between the primary tumor and lymph node metastases [36]. The differences with our study are related to a number of cases (Mimori et al. with 6 cases, Arun et al. and Kristek et al. with 60 cases each), method of markers assessment (cDNA microarray at Mimori et al.) and the most important, that all of them reported BCL2 expression from classical, histopathological position of breast cancer interpretation (34-36). A direct and comprehensive comparison from molecular position of BCL2 expression between primary breast carcinomas and paired distant metastases has not been performed yet.

In summary, molecular subtypes and BCL2 expression are not stable during tumor progression and metastatic development. In the present study we established immunohistochemically that BCL2 is not influenced by subtypes' transitions. BCL2 switches were encountered only in cases with a stable HER2, Luminal A or B phenotypes. Due to its dual function, promoting cell cycle arrest and preventing apoptosis, many things concerning BCL2 implication in breast cancer progression and drug resistance remain still unclear. Its specific localization at the crossroads of several complex physiological pathways, may lead to unexpected consequences, interesting for researches, dangerous for patients.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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