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Authors: Anna Liszcz-Tymoszuk, Marta Fudalej, Andrzej Deptała, Anna Maria Badowska-Kozakiewicz

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Cyclooxygenase-2 and Bcl-2 expression in patients with triple-negative breast cancer

Anna Liszcz-Tymoszuk[1, 2], Marta Fudalej[1], Andrzej Deptała[1], Anna M. Badowska-Kozakiewicz[1]

1. Department of Cancer Prevention, Medical University of Warsaw, Warsaw, Poland

2. Students' Scientific Organization of Cancer Cell Biology, Department of Cancer Prevention, Medical University of Warsaw, Warsaw, Poland

Introduction. Triple-negative breast cancer (TNBC) is a rare type of breast cancer associated with lack of expression of estrogen and progesterone receptors and HER2 protein, characterized by poor outcome and chemotherapy resistance. Cyclooxygenase-2 (COX-2) is a constitutional enzyme responsible for prostaglandin synthesis, present in neoplastic cells and premalignant lesions. B-cell lymphoma 2 (Bcl-2) protein is considered to be one of the most potent apoptosis-regulating agents, assuring body homeostasis.

Aim. The aim of the present study was to evaluate the immunohistochemical (IHC) profile of COX-2 and Bcl-2 expression in patients suffering from TNBC in order to obtain more detailed data on additional factors negatively influencing TNBC outcome.

Material and methods. The IHC evaluation of COX-2 and Bcl-2 expression among 21 women with diagnosis of TNBC was performed.

Results. The most common histological subtype was invasive ductal cancer of no special type. COX-2 was present in all examined samples with moderate to strong expression detected in 20 of 21 cases. There was a positive correlation between histological grade (G) and COX-2 expression (p=0.002). Bcl-2 was present in all examined samples. The analysis has shown that tumours presenting highy positive expression of Bcl-2 accounted for the majority of examined cases (57.2%).

Conclusions. Achieved results might lead to a conclusion that COX-2 and Bcl-2 high expression in TNBC may be linked with tumour aggressiveness and poor overall survival. However, before their consideration as additional markers used in routine histological examination and grading in breast cancer, further studies are necessary.

Key words: Bcl-2, cyclooxygenase-2, immunohistochemistry, prognostic factors, triple-negative breast cancer

Introduction

Breast cancer is the most common type of cancer among women worldwide. This heterogeneous group of malignant neoplasms represents 22.2% of newly diagnosed cancer cases and 13.3% of cancer-related deaths. Unfortunately, its incidence is constantly on the rise [1]. Triple-negative breast

cancer (TNBC) is a rare histological type of breast cancer characterized by lack of presence of estrogen and progesterone receptors and HER2 protein. Wide range of studies showed its association with poor outcome, low 5-year overall survival rate, chemotherapy resistance and co-existence with younger patients age [2].

Cyclooxygenase is a constitutional enzyme responsible for prostaglandin and thromboxane synthesis, occurring in two isoforms. Cyclooxygenase-2 (COX-2) is present in inflamed tissues, neoplastic cells and premalignant lesions. It enhances cellular proliferation, tissue invasion and angiogenesis, in addition to its anti-apoptotic effect [3], thus provides vital conditions for developing tumour. Epidemiological studies showed relation between COX-inhibiting drugs (nonsteroidal anti-inflammatory drugs – NSAIDs) and reduced cancer risk of gastrointestinal tract [3]. Studies conducted over the years showed that medications inhibiting COX-2 might act as possible chemopreventive agents in breast cancer, since increased expression of COX-2 in tumour samples was often observed [4, 5]. As a result of those findings cyclooxygenase was also considered as a biochemical marker of poor prognosis.

Another fundamental aspect of neoplastic processes is evasion of programmed cell death. B-cell lymphoma 2 (Bcl-2) protein is a product of *BCL-2* gene and is considered to be one of the most potent apoptosis-regulating agents, assuring body homeostasis. This protein prevents apoptosis by deterring cytochrome C and AIF (apoptosis inducing factor) in mitochondria, thus inhibiting the caspase-dependent apoptosis pathway [6]. Overexpression of Bcl-2 was observed in number of cancers; in case of breast cancer. Moreover, expression of Bcl-2 was established as an independent risk factor of worse prognosis of breast cancer [6, 13, 30].

The aim of our study was to evaluate immunohistochemical profile of COX-2 and Bcl-2 expression in patients suffering from TNBC in order to obtain more detailed data on additional factors negatively influencing TNBC outcome.

Material and methods

Patients

The patient population comprised 21 women with diagnosis of triple-negative breast cancer. The material came from biopsies, excisional biopsies and modified radical mastectomies. They were fixed in 10% buffered formalin phosphate, dehydrated by set of alcohols of increasing concentrations, embedded in in paraffin and cut into serial sections of 4µm thick. Then, samples were rehydrated and stained with haematoxylin and eosine, allowing to classify them according to WHO classification. Moreover, the samples allowed to evaluate histological grade (G), tumour grade (T), lymph node involvement (N) of given tumours. Additionally, the expression of receptors for estrogen (ER), progesterone (PR) and HER

2 receptors was assessed by means of immunohistochemical staining, using mouse monoclonal antibodies (DAKO: IR654. IR068 and K5204) and DAKO EnVision™ system for visualisation of results. Stain intensity was assessed by computed image analysis of number of stained nuclei per 1000 neoplastic cells.

Detection of COX-2

Cyclooxygenase expression was determined using Monoclonal Mouse Anti-Human COX-2 antibody. First, the samples were dewaxed using set of alcohols of decreasing concentrations. Then, they were put info pH 6 buffer and put into water bath for 30 minutes in 90°C for antigen retrieval.

Subsequently, the preparations were left at room temperature for 20 minutes. Then, samples were rinsed twice in distilled water and then incubated wit 3% hydrogen peroxide for 5 minutes in order to quench endogenous peroxidase activity. Furtherly, they were washed in TRIS (Tris-Buffered Saline, pH 8, SIGMA) and then incubated with primary antibody in humidity chamber for 60 minutes in room temperature. In the next stage, samples were again washed in TRIS for 10 minutes and incubated with visualisation reagent for 30 minutes. Next, after being washed in TRIS, were incubated with 3,3-diaminobenidine (DAB) for visualisation of staining' results. The time of incubation was controlled, in order to obtain desired stain intensity. At the end of the procedure, preparations were counter-stained with haematoxylin. Stain intensity was assessed by computed image analysis of number of stained cytoplasm per 1000 neoplastic cells. Following score was adapted, similarly as in Nam et al. and others' research [7, 18, 35]:

- none – less than 10% positively stained cells,
- weak – 10% positively stained cells,
- medium – from 10% to 30% positively stained cells,
- strong – over 30% positively stained cells.

Detection of Bcl-2

Bcl-2 expression was assessed using monoclonal mouse anti-human Bcl-2 antibody. After dewaxing, the samples were incubated for 10 minutes in 1% hydrogen peroxide diluted in PBS (Phosphate Buffer Saline) to quench endogenous peroxidase activity. Then, they were washed in PBS twice for 5 minutes. Next, they were incubated with 1.5% blocking serum in PBS for one hour in room temperature. Then they were incubated with primary antibody diluted (1:50) in 1.5% blocking serum in PBS for 30 minutes at room temperature, and then washed thrice with PBS. Furtherly, sections were incubated for 30 minutes with AB enzyme reagent (avidin + biotinylated rorseradish peroxidase – HRP) and then washed with three changes of PBS for 5 minutes each. At the end, samples were

incubated with 3 drops of peroxidase substrate for 5 minutes, until desired stain intensity developed. The process concluded with counterstain with haematoxylin. Stain intensity was assessed by computed image analysis of number of stained cytoplasm per 1000 neoplastic cells. The scoring method was modified from score used by van Slooten et al. [8] and others [32–34] in their research, to emphasise different levels of stain intensity, and adapted as followed:

- none – less than 10% positively stained cells,
- weak – from 10% to 50% positively stained cells,
- medium – from 50% to 80% positively stained cells,
- strong – from 80% to 100% positively stained cells.

Statistical analysis

All the results were obtained using SPSS v. 12.0 PL Windows and Statistica 13.1. Chi-square test, Fisher exact test were performed. Statistical significance was set at p = 0.05, however, for some of calculations p was set at 0.008 (0.05/6) because Bonferroni correction was used to counteract the problem of multiple comparisons. In order to establish relations between COX-2 levels, Bcl-2 levels and patients age Spearman rang test was performed. R value lesser then 0.2 is considered as without correlation.

Results

Pathological examination was performed on total of 21 female patients with confirmed diagnosis of TNBC. In the present study, we observed and analysed the expression and relationship of COX-2 and Bcl-2 with means of immunohistochemistry (tab. I).

14 out of 21 patients (66.7%) were above 50 years of age at the time of diagnosis (mean age 55.5 years old). The most common histological subtype was invasive ductal cancer of no special type (IDC-NST – 61.9%). Majority of samples were assessed as pT2 (57.1%). Lymph node involvement examination showed the dominance of pN1 stage, with 11 cases out of 21 (52.4%), followed by N0 (38.1%). Detailed pathological characteristics are included in Table I. Presence of distant metastases was not evaluated in the study.

Vast majority of examined tumours were assessed as moderately differentiated G2 (57.1%) and poorly differentiated G3 (38.1%), leaving only one sample with well differentiated cell architecture. Correlations between histological grade, tumour size and lymph node status were examined, with no statistically significant relations.

As shown in table II, COX-2 was present in all examined samples with moderate to strong expression detected in 20 of 21 cases (staining intensity of 2 and higher).

There was a positive correlation between histological grade (G) and COX-2 expression ($p = 0.002$). However, there was no statistically significant relationship between COX-2 presence, lymph node involvement (N) and type of neoplasms. Relation between patient age and COX-2 levels was also not significant ($R = 0.00$). Considering COX-2 expression, tumours were more likely to be identified as IDC-NST (tab. III).

Bcl-2 was present in all examined samples (tab. IV), demonstrating moderate and higher level of cytoplasmic expression in nearly half of them (staining intensity of 3 and higher – 12/21 of analysed specimens). No correlation was found between tumour stage, histological grade, lymph node involvement and the level of expression of Bcl-2 (tab. V). We identified no association between Bcl-2 expression and patients age ($R = 0.167$). The analysis has shown that tumours presenting positive expression of Bcl-2 (of staining intensity of 3 and higher) accounted for the majority of examined cases (57.2%) and were more likely to be assessed as T2, N1 and G2.

Discussion

Breast cancer is one of most frequently diagnosed neoplasms in developed countries, resulting in almost 15% of cancer-related deaths amongst women [1]. Triple-negative breast cancer is a very rare subtype of this type of cancer, characterized by the lack of expression of ER, PR and HER2, accounting for 15–20% of cases. Previous studies have shown that TNBC diagnosis is a negative prognostic factor in breast cancer [9, 10], as well as high COX-2 expression [11, 12] and Bcl-2 expression [13, 14]. Considering all the above, we aimed to obtain more detailed data on additional factors negatively influencing TNBC outcome. The goal of the present study was to evaluate immunohistochemical profile of COX-2 and Bcl-2 expression in patients suffering from TNBC.

COX-2 is known for its association with poor prognosis in breast cancer patients. In 2015 Xu et al. [14] conducted a meta-analysis including twenty-one studies with 6739 patients trying to evaluate prognostic value of COX-2 and its association with clinicopathological characteristics. Their study proved that expression of COX-2 predicts greater tumour size and presence of lymph node metastasis, whereas they indicated no significant correlation between ER, PR and HER2 status and COX-2 expression. The mechanism of detected association remained unclear and role of COX-2 in TNBC was not widely discussed and examined.

In the present study there was no statistically significant relation between COX-2 presence and lymph node involvement, nevertheless this correlation was found in many previously conducted studies [14]. Some researchers try to explain the mechanism of this correlation. In 2017 Krishnamachary et al. [15] investigated the role of COX-2 expression by TNBC cells in shaping the

structure and function of the tumour extra-cellular matrix (ECM), which may affect forming of metastasis. In their study COX-2 downregulation impacted ECM structure by reducing collagen I (Col1) fiber volume, which then resulted in a reduced ability of TNBC cells to metastasize to lymph nodes. Col1 fiber density and orientation were previously linked to breast cancer metastasis – in 2012 Kakkad et al. in their pilot study [16] revealed statistically significant increase of Col1 fiber density in breast cancers with lymph nodes involvement.

Our results showed that vast majority of TNBC cases were characterised by highly positive expression rate of COX-2 (95.2% of cases). In a study performed by Chikman et al. [17], only 57.4% of patients were classified as COX-2-positive. They found a prognostic significance of COX-2 for TNBC – 5-year disease-free survival rate reached 83.9% in COX-2-negative patients, whereas it was only 58.3% in COX-2-positive TNBC patients. No prognostic significance of COX-2 expression was proved for other types of breast cancer.

Molsapuria et al. investigated a cohort with similar clinicopathological characteristics (dominant T2, 31 TNBC cases), with positive association between COX-2 expression and both TNBC and high tumour grade, whereas in the present study the correlation was positive only with histological grade [18]. However, Zhau et al. [19] showed no correlation between any of clinicopathological characteristics. Similarly, Basudhar et al. [20] showed no correlation between COX-2 levels and histological grade. Chickman et al. [17] presented a lack of correlation between any hormonal receptor status and COX-2 expression, and our results are in accordance to those findings. On the other hand, Ristmaki et al. [21] showed positive correlation between COX-2 expression levels and negative hormone status, large tumour size, high histological grade, high proliferation rate (identified by Ki-67), high p53 expression, ductal type and axillary lymph node involvement, which is a well-known independent risk factor for the poor outcome [22]. In the present study positive nodal involvement was common, majority of which assessed as N1, with no statistical significance.

Simonnson et al. carried out one of largest studies evaluating COX-2 expression in breast cancer, where non-TNBC cancers were associated with high COX-2 expression, lower, less aggressive tumour characteristics and higher age [23]. Moreover, in their study TNBC correlated negatively with high COX-2 expression. In the present study, the results did not indicate any relation between age and moderate tumour malignancy.

Members of Bcl-2 family belong to a group of pivotal arbiters of mitochondria-mediated apoptosis, consisting of anti-apoptotic and pro-apoptotic members. The role of Bcl-2 in apoptosis regulation seems to be well established, however its role in tumorigenesis remains unclear. Changes in genome leading to overexpression of anti-apoptotic proteins like Bcl-2 or Bcl-xl are reported in wide range of malignancies, including breast cancer [24]. Paradoxically, Bcl-2 protein expression in breast cancer is associated with favourable phenotype of low-grade, ER positive, slowly proliferating

breast tumours and better prognosis [25]. What it more, Bcl-2 was established as a marker that could improve the prognostic power of Nottingham Prognostic Index [26].

One study found a correlation between increased COX-2 expression and Bcl-2 expression both in TNBC and non-TNBC patients [27]. The potential role of Bcl-2 as a prognostic factor for breast cancer has been examined in previous studies; nevertheless, its role in pathogenesis and course of TNBC needs further research.

The frequency of Bcl-2 overexpression in TNBC varies significantly. In the present study all examined samples presented strong Bcl-2 expression (of score 2 and higher), whereas Escórcio-Dourado et al. observed it in 40% of 30 studied cases [28]. In 2013 Abdel-Fatah et al. described Bcl-2 as an independent prognostic marker of TNBC [29]. They observed a positive expression of Bcl-2 in 29.8% of examined samples. Moreover, it was significantly associated with high expression of p27, MDM4 and SPAG5. Taking into consideration only Bcl-2-positive group they found that G2 and G3 made up the largest percentage of cases – similarly to the present study. As far as tumour size is concerned, they observed T2 stage in 44.1% of cases comparable to 57.1% of studied cases – in both studies T2 tumours accounted for the largest group. Their study proved that loss of Bcl-2 considerably escalates the risk of both death and recurrence in TNBC.

In the study conducted by Abd El-Hafez et al. on a similar group of patients with TNBC, they observed Bcl-2 positive staining in 85% of invasive ductal carcinomas [30]. It is worth mentioning that they reported opposite results concerning patients age and grading of the tumours. In the present study 66.7% patients were above 50 years old at the time of diagnosis, whereas Abd El-Hafez et al. reported that Bcl-2 was more frequently expressed in younger patients, accounting for 81.3% of cases. Moreover, they correlated Bcl-2 expression with lower grading whereas in the present research we did not observe the group of G0. These contradictory statements lead us to conclusion that role of Bcl-2 and its prognostic value in TNBC still seems unclear and needs further research on wider group of patients.

All mentioned above may lead to a conclusion that COX-2 and Bcl-2 high expression in triple-negative breast cancer may be an interesting asset in routine histological examination and grading of breast cancer, however further studies with greater group of specimen are necessary. Moreover, as they are usually present in higher graded neoplasms, COX-2 and Bcl-2 may also serve as potential new targets for systemic treatment. This approach could potentially reveal new methods in the therapy of triple-negative breast cancer. This is crucial, as hormonotherapy and HER2 targeting remain unavailable for those patients. Described association should be investigated further, as the group of patients was small, even though representing a rare histological subtype of breast cancer.

Conflict of interest: none declared

Anna Liszcz-Tymoszuk

Medical University of Warsaw

Department of Cancer Prevention

ul. Erazma Ciołka 27

01-445 Warszawa, Poland

e-mail: aliszcz@hotmail.com

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Table I. Clinicopathological characteristics of the patients included in the study

Characteristics	**Number of patients, (%) (n = 21)**
mean age, years	55.5
age	
• under 50 y.o.	7 (33.3%)
• above 50 y.o.	14 (66.7%)
histological type	
• IDC	6 (28.6%)
• IDC-NST	13 (61.9%)
metaplastic	2 (9.5)%
tumour size (T)	
• T1	4 (19.1%)
• T2	12 (57.1%)
• T3	3 (14.3%)
• T4	2 (9.5%)
lymph node involvement (N)	
• N0	8 (38.1%)
• N1	11 (52.3%)
• N2	1 (4.8%)
• N3	1 (4.8%)
histological grade (G)	
• G1	1 (4.8%)
• G2	12 (57.1%)
• G3	8 (38.1%)
COX-2 expression	
• 0	0
• 1	1 (4.8%)
• 2	9 (42.8%)
• 3	11 (52.4%)
Bcl-2 expression	
• 0	0
• 1	0
• 2	9 (42.8%)
• 3	6 (28.6%)
• 4	6 (28.6%)

Bcl-2 – B-cell lymphoma 2; COX-2 – cyclooxygenase 2; IDC – invasive ductal carcinoma, IDC-NST – invasive ductal carcinoma – no special type; y.o. – years old

Table II. IHC staining of COX-2 in studied samples

Percentage and degree of positively stained cells			**TNBC samples with positive reaction**
>10%	1	none	0
10%	2	weak	1 (4.8%)
>10–30%	3	medium	9 (42.9%)
>30%	4	strong	11 (52.3%)

COX-2 – cyclooxygenase 2; IHC – immunohistochemical; TNBC – triple negative breast cancer

Table III. Relations between degree of COX-2 staining and clinicopathological features

	Degree of immunohistochemical expression of COX-2 in TNBC samples				
	None	**Weak**	**Medium**	**Strong**	**p**
Histological type					
IDC-NST	0	1 (4.8%)	6 (28.6%)	6 (28.6%)	0.889
IDC	0	0	3 (14.3%)	3 (14.3%)	
METAPLASTIC	0	0	1 (4.8%)	1 (4.8%)	
G1	0	1 (4.8%)	0	0	0.002
G2	0	0	7 (33.5%)	5 (23.8%)	
G3	0	0	6 (28.6%)	2 (9.5%)	
T1	0	0	2 (9.5%)	2 (9.5%)	0.828
T2	0	1 (4.9%)	5 (23.8%)	6 (28.6%)	
T3	0	0	2 (9.5%)	1 (4.8%)	
T4	0	0	0	2 (9.5%)	
N0	0	1 (4.8%)	1 (4.8%)	6 (28.6%)	0.130
N1	0	0	6 (28.6%)	5 (23.8%)	
N2	0	0	1 (4.8%)	0	
N3	0	0	1 (4.8%)	0	

COX-2 – cyclooxygenase 2; IDC – invasive ductal carcinoma; IDC-NST – Invasive ductal carcinoma – no special type; TNBC – triple negative breast cancer

Table IV. IHC staining of Bcl-2 in studied samples

Percentage and degree of positively stained cells			**TNBC samples with positive reaction**
<10%	1	none	0
10–50%	2	weak	9 (42.8%)
50–80%	3	medium	6 (28.6%)
>80%	4	strong	6 (28.6%)

Bcl-2 – B-cell lymphoma 2; IHC – Immunohistochemical; TNBC – triple negative breast cancer

Table V. Relations between degree of Bcl-2 staining and clinicopathological features

	Grade of IHC expression of Bcl-2 in TNBC samples				
	None	**Weak**	**Medium**	**Strong**	**p**
Histologic type					
IDC-NST	0	4 (19.0%)	4 (19.0%)	5 (23.8%)	0.522
IDC	0	4 (19.0%)	1 (4.8%)	1 (4.8%)	
metaplastic	0	1 (4.8%)	1 (4.8%)	0	
G1	0	0	0	0	1.0
G2	1 (4.8%)	5 (23.8%)	3 (14.3%)	4 (19.0%)	
G3	0	3 (14.3%)	3 (14.3%)	2 (9.5%)	
T1	0	3 (14.3%)	0	1 (4.8%)	0.828
T2	0	4 (19.0%)	5 (23.8%)	3 (14.3%)	
T3	0	1 (4.8%)	1 (4.8%)	1 (4.8%)	
T4	0	1 (4.8%)	0	1 (4.8%)	

N0	0	5 (23.8%)	2 (9.5%)	1 (4.8%)	0.610
N1	0	4 (19.0%)	3 (14.3%)	4 (19.0%)	
N2	0	0	1 (4.8%)	0	
N3	0	0	0	1 (4.8%)	

Bcl-2 – B-cell lymphoma 2; IDC – invasive ductal carcinoma; IDC-NST – invasive ductal carcinoma – no special type; IHC – immunohistochemical; TNBC – triple negative breast cancer.

Figure 1. Histopathological image of invasive triple negative breast cancer (TNBC) (H&E): left (A) – positive immunohistochemical staining for Bcl-2 (original magnification 200×); right (B) – positive immunohistochemical staining for Cox-2 (original magnification 1000×)