RESEARCH ARTICLE

BCL2 is an independent predictor of outcome in basal-like triple-negative breast cancers treated with adjuvant anthracycline-based chemotherapy

Katerina Bouchalova · Marek Svoboda · Gvantsa Kharaishvili · Jana Vrbkova ·
Jan Bouchal · Radek Trojanec · Vladimira Koudelakova · Lenka Radova ·
Karel Cwiertka · Marian Hajduch · Zdenek Kolar

Abstract Neither targeted therapies nor predictors for chemotherapy sensitivity are available for triple-negative breast cancer (TNBC). Our study included 187 patients with TNBC, 164 of whom were treated with anthracycline-based adjuvant chemotherapy. Eleven molecular biomarkers were analyzed. BCL2, epidermal growth factor receptor (EGFR), MYC, TOP2A, and Ki-67 protein expression was evaluated by immunohistochemistry. The status of the EGFR, MYC, and TOP2A genes and chromosomes 7, 8, and 17 was assessed using fluorescence in situ hybridization. High BCL2 expression predicted poor relapse-free survival (RFS) in patients treated with anthracycline-based adjuvant chemotherapy ($p=0.035$), poor breast cancer-specific survival (BCSS) ($p=0.048$), and a trend to poor overall survival (OS) ($p=0.085$). High levels of BCL2 expression predicted poor OS in basal-like (BL) TNBC patients treated with adjuvant anthracycline-based regimens (log-rank $p=0.033$, hazard ratio (HR) 3.04, 95 % confidence interval (CI) 1.04–8.91) and a trend to poor RFS (log-rank $p=0.079$) and poor BCSS (log-rank $p=0.056$). Multivariate analysis showed that BCL2

K. Bouchalova (✉) · J. Vrbkova · R. Trojanec · V. Koudelakova ·
L. Radova · M. Hajduch
Laboratory of Experimental Medicine, Institute of Molecular and
Translational Medicine, Faculty of Medicine and Dentistry, Palacky
University, Hnevotinska 5, 775 15 Olomouc, Czech Republic
e-mail: katerina.bouchalova@upol.cz

K. Bouchalova
Department of Pediatrics, Faculty of Medicine and Dentistry, Palacky
University, University Hospital Olomouc, Puskinova 6,
775 20 Olomouc, Czech Republic

M. Svoboda
Masaryk Memorial Cancer Institute, Zluty kopec 7,
656 53 Brno, Czech Republic

G. Kharaishvili · J. Bouchal · Z. Kolar
Department of Clinical and Molecular Pathology, Institute of
Molecular and Translational Medicine, Faculty of Medicine and
Dentistry, Palacky University, Hnevotinska 3,
Olomouc, Czech Republic

K. Cwiertka
Department of Oncology, Faculty of Medicine and Dentistry, Palacky
University and University Hospital Olomouc, I.P. Pavlova 6,
775 20 Olomouc, Czech Republic

status, tumor size, and nodal status all had independent predictive significance for RFS (p=0.005, *p*=0.091, *p*=0.003, respectively; likelihood ratio test for the whole model, *p*= 0.003), BCSS (*p*=0.012, *p*=0.077, *p*=0.01, respectively; likelihood ratio test for the whole model, *p*=0.016), and OS (*p*= 0.008, *p*=0.004, *p*=0.004, respectively; likelihood ratio test for the whole model, *p*=0.0006). Similarly, multivariate analysis for BL TNBC showed BCL2, tumor size, and nodal status all had independent predictive significance for RFS (likelihood ratio test for the whole model, *p*=0.00125), BCSS (*p*= 0.00035), and OS (*p*=0.00063). High EGFR expression was associated with poor BCSS (*p*=0.039) in patients treated with anthracycline-based adjuvant chemotherapy. Patients who underwent anthracycline-based adjuvant chemotherapy and exhibited CMYC amplification had a trend to worse BCSS (*p*=0.066). In conclusion, high BCL2 expression is a significant independent predictor of poor outcome in TNBC patients treated with anthracycline-based adjuvant chemotherapy, and this is the first study showing the BCL2 prediction in BL TNBC. BCL2 expression analysis could facilitate decision making on adjuvant treatment in TNBC patients.

Introduction

Triple-negative breast cancer (TNBC) is a clinically and molecularly heterogeneous disease [1–3]. It has the highest frequency of somatic rearrangements among breast carcinomas (BCs) [4] and encompasses at least seven subtypes with different prevalences, gene expression profiles, and clinical outcomes [1, 2]. Although it is generally a fast-growing and highly malignant disease, its frequent sensitivity to chemotherapy makes it potentially curable if detected at a sufficiently early stage. On the other hand, experiences with neoadjuvant and adjuvant treatment indicate that approximately one third of TNBC cases are primarily chemoresistant, even when using regimes based on the most effective cytostatics such as anthracyclines, platinum derivatives, and taxanes. Because there is a lack of targeted treatments for TNBC in current clinical practice, there is an urgent need to identify molecular biomarkers that can be used to select appropriate chemotherapeutic regimens for a given cancer [1, 5–8].

BCL2 is a fundamental anti-apoptotic protein that plays an important role in physiological and pathological processes as a protooncogene and oncogene. It also performs other functions, such as promoting cell growth and proliferation [9–11]. Preclinical data showed that BCL2 expression was associated with resistance to anthracycline doxorubicin [12]. In keeping with this finding, the absence of BCL2 expression

in prechemotherapy samples was associated with a higher probability of pathological complete response (pCR) to neo-adjuvant doxorubicin-based chemotherapy [13–15]. In the context of adjuvant therapy, BCL2-negative patients treated with anthracyclines had better outcomes than those who received either cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) or no chemotherapy [15]. However, other studies have identified a positive association between prognosis and BCL2 protein expression in patients with breast cancer, including TNBC subgroups [9, 15]. It is not entirely clear why this is the case, but it may be partly due to changes in the expression of other proteins such as p27, HER3 and MDM4 in BCL2-positive cancers [15].

Epidermal growth factor receptor (EGFR, also known as HER1) plays roles in cell proliferation, migration, and protection against apoptosis [16]. Its overexpression appears to be a later event in tumorigenesis [17] and is frequently observed in TNBC, especially in metaplastic cancers where it occurs in up to 80 % of all cases [18]. The EGFR gene is amplified in 0–14 % of non-selected BC series and up to 28 % of metaplastic cancers [18]. However, the available data on the use of the EGFR oncogene/protein as a prognostic marker in BC are inconsistent and little is known about its potential utility as a marker in TNBC. Two studies found significant correlations between EGFR immunoreactivity (as a percentage of positive cells) and worse prognosis in both invasive ductal TNBC [19] and unselected TNBC [20].

The TOP2A gene encodes topoisomerase II alpha, the molecular target of the anthracyclines. Preclinical results indicate that TOP2A amplification may be predictive of anthracycline sensitivity [21]. Clinical evaluations of this relationship have yielded somewhat inconsistent results, but a large study of 4, 943 patients identified TOP2A and HER2 coamplification as a predictor of responsiveness to anthracycline-based chemotherapy [22]. TOP2A gene amplification is frequently observed in HER2-amplified BC and also in a small proportion (3–9 %) of HER2 non-amplified BC [18, 23]. At present, little is known about the role of TOP2A in TNBC.

Of the many proteins that have been examined as markers for TNBC, Ki-67 and CMYC merit particular attention. Ki-67 is a marker of proliferating cells [24], and Ki-67 labeling values above 35 % were recently shown to be associated with an increased 5-year cumulative incidence of breast-related death in a study of node-negative TNBC patients treated with either adjuvant therapy (75 % were treated with CMF and 20 % received anthracycline therapy) or no chemotherapy (14 %) [25]. CMYC encodes a multifunctional nuclear phosphoprotein that contributes to cell cycle progression, apoptosis, and cellular transformation. The CMYC gene is frequently altered in BC [18], but its role in TNBC is not well defined.

Anthracycline-based regimens are most frequently used to treat TNBC. However, some patients do not benefit from this therapy and predictors of sensitivity are urgently needed. First,

TOP2A encodes topoisomerase alpha, a target of anthracyclines, and changes in TOP2A gene/protein could influence the sensitivity to anthracyclines. Second, EGFR, a potential predictor for targeted therapy with tyrosine kinase inhibitors, has been assessed with respect to its expression in high proportion of TNBC patients. Importantly, neither TOP2A nor EGFR has been reported as predictors in TNBC. Finally, in case of Ki-67, the aim was to validate data from the above-mentioned study [25]. The objective of this work was to determine whether BCL2 or the other markers discussed above can predict outcomes in TNBC patients treated with adjuvant anthracycline-based regimens.

Materials and methods

Patients

A retrospective study was performed according to the RE-MARK criteria [26]. A consecutive series of 335 TNBC patients was diagnosed or treated at the Masaryk Memorial Cancer Institute between the years 2004 and 2009. The study included 187 patients with TNBC, 178 of whom were treated with adjuvant chemotherapy and 9 of whom received no systemic chemotherapy. The characteristics of the patient population treated with anthracycline-based adjuvant therapies ($n=$ 164) are summarized in Table 1. Patients treated with other chemotherapies ($n=17$) received taxane therapy ($n=11$) or CMF treatment ($n=3$). Clinical data were reviewed retrospectively from medical records, and follow-up was assessed prospectively. Patient data obtained during routine diagnostics, treatment, and follow-up were archived and anonymized, including the following: date of birth, date of histological diagnosis, age at diagnosis, number and type of tumors, clinical stage, tumor serology markers at diagnosis, performance status according Eastern Cooperative Oncology Group (ECOG) criteria, type of surgery, onset and termination of treatment, chemotherapeutic regimen, number of chemotherapy cycles, use of radiotherapy, date of therapy effect evaluation and effect of primary therapy, date of relapse, relapse localization, further therapy, date of last follow-up control, clinical status at the time of last follow-up, and cause of death for dead patients. Data on pTNM; histological tumor type; grading; and the status of the estrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor receptor 2 (HER2) were obtained from histological assessments of the patients' primary tumors. The study was approved by the University Hospital Ethics Committee.

Specimen characteristics and assay methods

Both immunohistochemistry (IHC) evaluations and fluorescence in situ hybridization (FISH) were performed on

Table 1 Patients' clinicopathological characteristics and BCL2 expression status

Characteristic		N	%
T	1	70	42.7
	2	85	51.8
	3	3	1.8
	4	5	3.0
	NA	1	0.6
N	0	99	60.4
	1	43	26.2
	2	10	6.1
	3	9	5.5
	NA	3	1.8
Stage	1	48	29.3
	2	93	56.7
	3	23	14.0
Grade	2	9	5.5
	3	155	94.5
Histopathological type	IDC	119	72.6
	ILC	3	1.8
	Medullary IC	22	13.4
	Metaplastic IC	6	3.7
	IC other subtypes	9	5.5
	IC non-differentiated	5	3.0
BCL2 histoscore	High	66	40.2
	Low	83	50.6
	NA	15	9.1
Radiotherapy	0	36	22.0
	1	128	78.0
Chemotherapy	Anthracycline-based	164	100.0
Anthracycline-based chemotherapy and BCL2 histoscore	Without taxane and high BCL2	46	28.0
	Without taxane and low BCL2	51	31.1
	With taxane and high BCL2	20	12.2
	With taxane and low BCL2	32	19.5
	NA	15	9.1

NA not available; *IC* invasive carcinoma; *IDC* invasive ductal carcinoma; *ILC* invasive lobular carcinoma; *IC* other subtypes: papillary, micropapillary, apocrine

formalin-fixed paraffin-embedded (FFPE) samples with a thickness of 4 μm as described previously [27, 28].

The expression of the HER2 protein was determined by the DAKO HercepTest (DAKO, Denmark) and scored on a qualitative scale from 0 to 3+ according to the DAKO manual and the guidelines for HER2 testing in BC published by the American Society of Clinical Oncology/College of American Pathologists. HER2 gene status was evaluated by FISH using the PathVysion HER2 kit (Abbott Laboratories, USA). The HER2 gene status was considered to be negative (FISH non-amplified) in cases where the HER2 gene/centromere of chromosome 17 ratio was less than 1.8. An IHC score of 0/1+ was

considered indicative of HER2 negativity at the protein level. The status of the estrogen receptor alpha (ERα) and progesterone receptor (PgR) was examined by IHC using SP1 and SP2 monoclonal rabbit antibodies (Lab Vision Thermo Fisher Scientific, USA). All but three of the cancers included in our study had 0 % expression of ERα and PgR.

Basal-like (BL) subgroup was defined according Nielsen et al. [29] as cytokeratin 5/6+ and/or EGFR+.

IHC was also used to study the expression of the following proteins (the sources of the antibodies used in each case are reported in parentheses): BCL2 (mouse, Biogenex, USA), EGFR (mouse, Neomarkers, USA), TOP2A (mouse, Dako, Denmark), Ki-67 (MIB1, Cell Signaling Technology, USA), and CMYC (mouse, Novocastra, UK). Proportion scores (0–100 %) and staining intensities (0–3) were evaluated, and histoscores were calculated as described previously. BCL2 expression was assessed as high (histoscore >10) or low (histoscore ≤10). EGFR expression was assessed as high (histoscore >120) or low (histoscore ≤120). The same cutoff was used for TOP2A protein expression. For CMYC, high and low expressions were indicated by histoscores of >190 and ≤190, respectively. Ki-67 positivity was analyzed using three different dichotomizing thresholds: 14 %, the median for the studied samples, and 35 %.

FISH was used to assess EGFR/HER1 gene amplification (Orange, 7p12, Intellmed) in relation to chromosome 7 (CEP 7, Green, Intellmed), TOP2A gene amplification (Orange, 17q21–q22, Intellmed) in relation to chromosome 17 (CEP 8, Green, Intellmed), and CMYC gene expression (Orange, 8q24, Intellmed) in relation to chromosome 8 (CEP 17, Green, Intellmed). Gene/chromosome ratios were calculated for each gene and chromosome pair. Ratios of ≥1.5 and <0.8 were considered indicative of gene amplification and deletion, respectively. Gene copy numbers of >4 were also considered indicative of gene amplification. Patients were grouped into four categories based on the percentage of cells in their tumors exhibiting gene amplification or deletion: those for whom >30 % of all tumor cells had one gene copy were assigned to group "−1," those in which >50 % of all tumor cells had three or more gene copies were assigned to the "+1" group, those having five or more gene copies were assigned to the "+2" group, and those in group "0" had normal gene status.

Study design and statistics

All laboratory assessments were performed in a blind manner, and clinical and laboratory data were subsequently combined. The endpoints were relapse-free survival (RFS), breast cancer-specific survival (BCSS), and overall survival (OS). RFS was defined as the length of time from the surgery to any relapse or death, whichever occurred first. BCSS was assessed as the time from surgery to the date of death from breast cancer or the date of last follow-up. OS was determined as the time from surgery until the date of death (from any cause) or the date of last follow-up.

STATISTICA 12.0 and R [30] were used for the statistical analyses (Spearman coefficient, Kaplan-Meier survival analysis, log-rank test, multivariate backward stepwise Cox analysis).

Results

High BCL2 expression predicts poor anthracycline treatment outcome

Table 1 shows the distribution of patients with low and high levels of BCL2 expression as determined by IHC staining (Fig. 1). High levels of BCL2 protein expression predicted poor RFS (log-rank $p=0.035$, HR 2.37, 95 % CI 1.04–5.42) (Fig. 2a) and poor BCSS (log-rank $p=0.048$, HR 2.63, 95 % CI 0.97–7.12) (Fig. 2b) in patients treated with adjuvant anthracycline-based regimens. High BCL2 expression also predicted a trend to poor overall survival (OS) in these patients (log-rank $p=0.085$, HR 2.15, 95 % CI 0.88–5.27) (Fig. 2c). In addition, stage (RFS $p=0.0004$, OS $p=0.0002$), tumor size (RFS $p=0.003$, OS $p=4e{-}7$), and nodal status (RFS $p=0.018$, OS $p=0.016$) were associated with outcome in a univariate analysis of anthracycline-treated patients. Low BCL2 expression had high negative predictive values (NPV) for relapse-free survival (89.16 %; 95 % CI 80.66–94.19 %) and OS (90.36 %; 95 % CI 82.12–95.03 %). Multivariate analysis revealed that BCL2 expression, tumor size, and nodal status all had independent predictive significance for RFS ($p=0.005$, $p=0.091$, and $p=0.003$, respectively; likelihood ratio test for the whole model, $p=0.003$), BCSS ($p=0.012$, $p=0.077$, $p=0.01$, respectively; likelihood ratio test for the whole model, $p=0.016$), and OS ($p=0.008$, $p=0.004$, $p=0.004$, respectively; likelihood ratio test for the whole model, $p=0.0006$). The initial multivariate analysis model included BCL2 status, age, tumor size, nodal status, and histopathological grade as variables (Supplementary Table S1A). Detailed information on age and follow-up is shown in Supplementary Table S2.

High BCL2 expression predicts poor anthracycline treatment outcome in BL TNBC

High levels of BCL2 protein expression predicted poor OS in BL TNBC patients treated with adjuvant anthracycline-based regimens (log-rank $p=0.033$, HR 3.04, 95 % CI 1.04–8.91) (Fig. 2f). High BCL2 expression also predicted a trend to poor RFS (log-rank $p=0.079$, HR 2.57, 95 % CI 0.86–7.68) (Fig. 2d) and poor BCSS (log-rank $p=0.056$, HR 2.78, 95 % CI 0.93–8.3) (Fig. 2e). Multivariate analysis revealed that BCL2 expression, tumor size, and nodal status all had
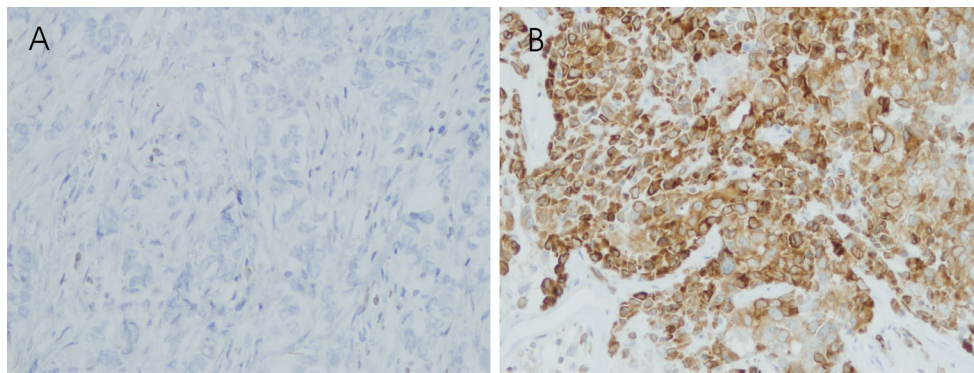
Fig. 1 Immunohistochemical staining (IHC) of the BCL2 protein (mouse antibody, Biogenex, USA) in formalin-fixed paraffin-embedded (FFPE) triple-negative breast cancer tissue samples. **a** BCL2 low (invasive ductal carcinoma, grade 3, histoscore 0, magnification ×200). **b** BCL2 high (medullary carcinoma, grade 3, histoscore 180, cytoplasmic staining, magnification ×200)

independent predictive significance for RFS ($p=0.029$, $p=0.013$, and $p=0.003$, respectively; likelihood ratio test for the whole model, $p=0.00125$), BCSS ($p=0.017$, $p=0.0106$, $p=0.002$, respectively; likelihood ratio test for the whole model, $p=0.00035$), and OS ($p=0.007$, $p=0.0236$, $p=0.0029$, respectively; likelihood ratio test for the whole model, $p=0.00063$). The initial multivariate analysis model included BCL2 status, age, tumor size, nodal status, and histopathological grade as variables (Supplementary Table S1B). The non-BL TNBC group was too small to obtain significant associations (Supplementary Table S1C).

EGFR protein expression and survival

High EGFR expression was present in 20 of the 148 (13.5 %) patients treated with anthracycline-based chemotherapy and 21 of all 166 (12.7 %) patients in our series. High EGFR protein expression was associated with a high probability of breast cancer-associated death in patients treated with anthracycline-based adjuvant chemotherapy (log-rank $p=0.039$, HR 2.87, 95 % CI 1.01–8.15) (Fig. 3). However, in a multivariate model including tumor size, nodal status, age, grade, and EGFR expression as variables, only nodal status had borderline independent predictive significance ($p=0.044$) for BCSS (log-rank test for the whole model, $p=0.055$). High EGFR protein expression was associated with a trend to worse BCSS and OS in all patients (log-rank $p=0.077$ and $p=0.086$, respectively) (Supplementary Figs. S1A and S1B). Again, EGFR expression was not an independent factor for BCSS or OS in this multivariate analysis. EGFR status was not associated with RFS in either anthracycline-treated patients or the whole series.

Other markers in prognosis and prediction

Patients were considered to exhibit CMYC amplification if they had a CMYC/chromosome 8 ratio of ≥1.5 and a CMYC copy number of ≥4. Ratios and copy numbers indicative of

CMYC amplification were identified in 13/143 (9.1 %) and 23/143 (16.1 %) of anthracycline-treated patients, respectively. CMYC copy numbers of ≥4 were associated with a trend to worse RFS ($p=0.066$) and BCSS ($p=0.082$) in anthracycline-treated patients (Supplementary Figs. S2A and S2B). The levels of CMYC amplification in all patients were similar to those for the anthracycline-treated cohort: 14/160 (8.8 %) exhibited a CMYC/chromosome 8 ratio of ≥1.5, while 27/160 (16.9 %) exhibited a CMYC copy number of ≥4. The association between CMYC amplification and a trend to worse BCSS ($p=0.087$) was also detected in the complete set of patients (Supplementary Fig. S2C).

Patients were considered to exhibit TOP2A amplification if they had a TOP2A/chromosome 17 ratio of ≥1.5 and a TOP2A copy number of ≥4. Ratios and copy numbers indicative of TOP2A amplification were found in 3/143 (2.1 %) and 8/143 (5.6 %) of anthracycline-treated patients, respectively. The corresponding figures for all patients were 4/160 (2.5 %) and 9/160 (5.6 %), respectively. TOP2A amplification was not associated with any of the considered outcomes (RFS, BCSS, or OS). Only one tumor exhibited a TOP2A deletion, indicated by a TOP2A/chromosome 17 ratio of <0.8.

EGFR gene amplification, indicated by an EGFR/chromosome 7 ratio of ≥1.5 and an EGFR copy number of ≥4, was rare in both anthracycline-treated patients and all patients. Of the anthracycline-treated group, 6/143 (4.2 %) exhibited a high EGFR/chromosome 7 ratio and 11/143 (7.7 %) exhibited a high EGFR copy number. The corresponding values for the whole series were 7/160 (4.4 %) and 12/160 (7.5 %), respectively. Neither EGFR amplification nor changes in the numbers of chromosomes 7, 8, or 17 were associated with any survival outcome.

All but two of the patients exhibited >14 % staining for the proliferation marker Ki-67, demonstrating the high proliferative capacity of TNBC. Ki-67 staining was analyzed as both a categorical and continual variable. However, neither analysis revealed any association between Ki-67 staining and survival

A

B

C

D

E

F
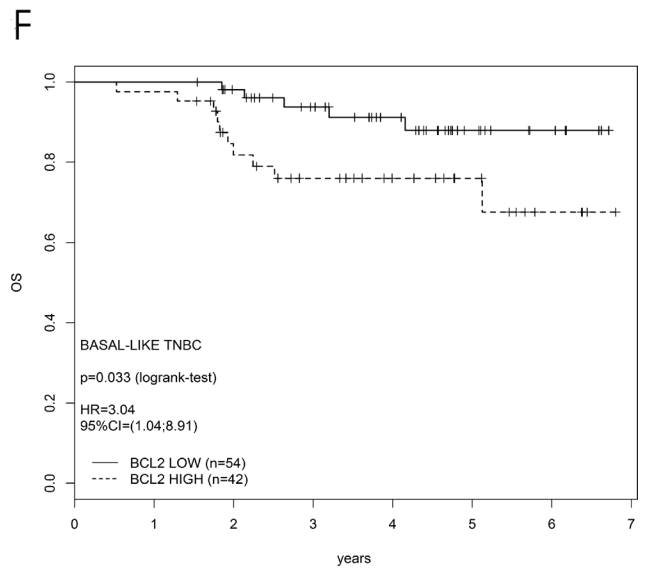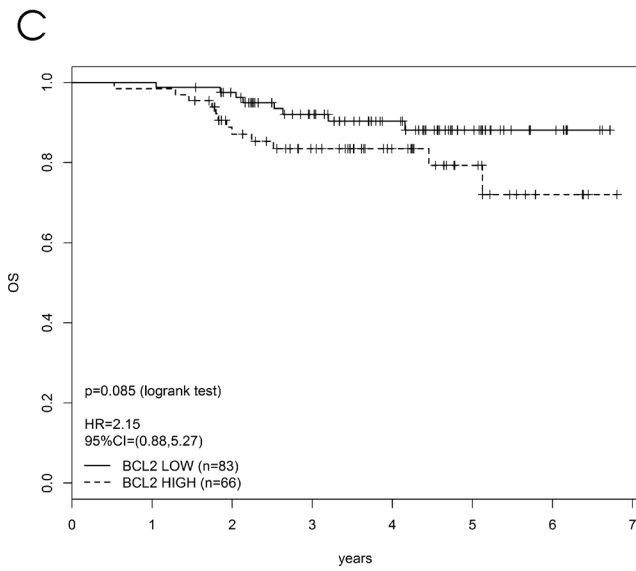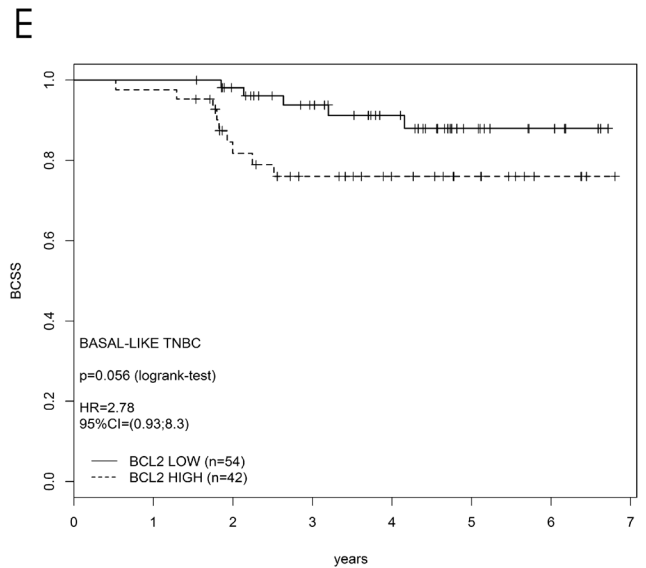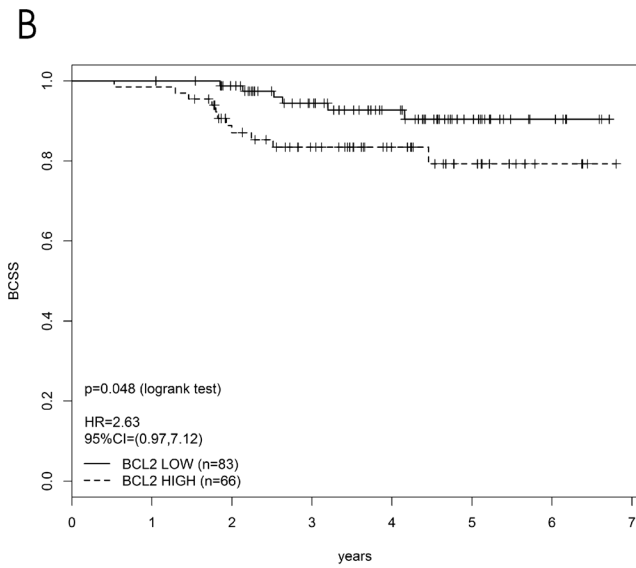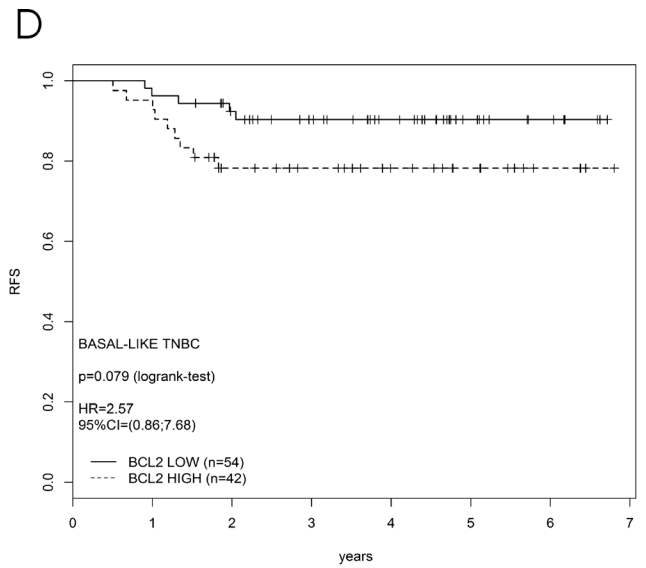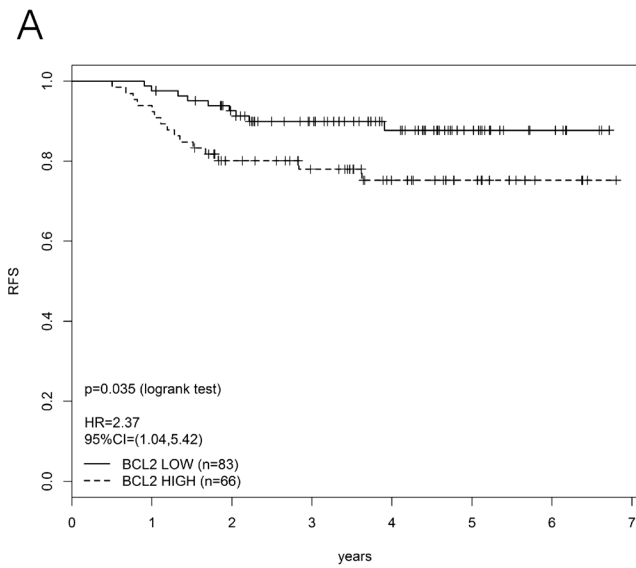
◀ **Fig. 2** Kaplan-Meier curves for BCL2. High BCL2 expression significantly associates with poor relapse-free survival (RFS) (**a**), with poor breast cancer-specific survival (BCSS) (**b**), and with a trend to worse overall survival (OS) (**c**) in the whole cohort of TNBC treated with anthracycline-based adjuvant chemotherapy. A trend to worse RFS (**d**), BCSS (**e**), and significant association with poor OS (**f**) was found for high BCL2 expression in basal-like TNBC subgroup treated with anthracycline-based adjuvant chemotherapy. Low BCL2 expression was used as a reference for hazard ratio in all graphs (HR 1.00)

outcomes (RFS, BCSS, or OS). Similarly, the expression of the TOP2A and CMYC proteins was also not associated with any survival outcome.

Discussion

We found an association between high BCL2 protein expression and poor outcome in TNBC patients treated with adjuvant anthracycline-based chemotherapies (this study and [31]). This treatment probably induces apoptotic cell death in tumors with low BCL2 expression. However, high levels of BCL2 expression block apoptosis, allowing the tumor cells to survive. This conclusion is supported by the observation that anti-BCL2 therapy (ABT-737) induced characteristics of apoptosis in vitro when applied in conjunction with the topoisomerase II poison etoposide [11]. An association between low levels of BCL2 expression and an enhanced anthracycline response (pCR) in neoadjuvant settings has also been reported by others [13, 15, 32]. In vitro, anti-BCL2 therapy involving

treatment with a BCL2 antisense oligonucleotide known as G3139 (oblimersen) increased the MDA-MB-231 cell line's chemosensitivity to anthracyclines and taxanes [12]. However, phase I/II clinical studies examining G3139 in combination with doxorubicin and docetaxel yielded unfavorable results [33]. The authors suggested that this failure may have been due to inadequate drug delivery to the tumor. BCL2 messenger RNA (mRNA) enrichment was observed in the mesenchymal stem-like TNBC subtype [1]. In a subsequent study, only 23 % (3/13) of patients with this TNBC subtype exhibited a pCR after neoadjuvant anthracycline/taxane chemotherapy [7].

We identified low BCL2 protein expression as an independent predictor of good outcome in patients treated with anthracycline-based adjuvant chemotherapy on the basis of a multivariate analysis using an initial model whose variables included age, tumor size, nodal status, and histopathological grade. Conversely, Abdel-Fatah et al. [15] found an association between BCL2 negativity and worse survival in early primary TNBC (*n*=635). This association was independent of chemotherapy (assessed as yes or no, where yes means that the patient was receiving some form of chemotherapy and no means that they were not), traditional pathological prognostic factors (lymph node stage, tumor size, tumor grade), and other potential confounders (lymphovascular invasion, mitotic index). Interestingly, the patients studied by Abdel-Fatah et al. were treated with a range of different chemotherapies including anthracycline-based regimens (*n*=177) and CMF (*n*=104). In addition, many (*n*=354) had no chemotherapy because they either declined systemic therapy or were diagnosed and treated before TNBC was identified as an aggressive

Fig. 3 Kaplan-Meier curve for EGFR. High EGFR protein expression associates with poor breast cancer-specific survival (BCSS) in TNBC patients treated with anthracycline-based chemotherapy
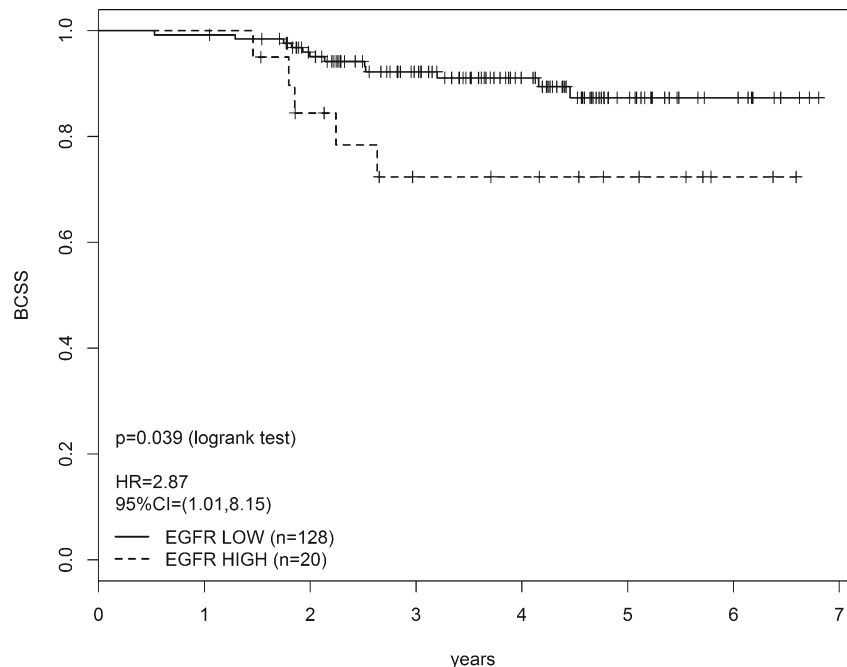
tumor entity or before adjuvant chemotherapy became the standard of care. Importantly, BCL2-negative patients treated with anthracycline-containing adjuvant therapies had better survival than those treated with adjuvant CMF or no systemic chemotherapy. However, there was no significant difference in survival between patients with tumors having high and low BCL2 expressions. On the other hand, positive BCL2 protein expression was associated with good outcomes in TNBC patients treated with CMF [15, 34]. Recently, similar data were published in unselected BC [35].

BCL2 as a predictor of outcome in non-BL TNBC was recently reported by Choi et al. [36]. We could not confirm this association, probably due to small number of non-BL TNBC patients. On the other hand, Choi et al. did not find BCL2 as a predictor in BL group. Inconsistent results could be caused also by different populations studied and TNBC heterogeneity itself. Moreover, another BCL2 antibody (clone 100) was used by Choi et al., while we used the same clone as in vast majority of publications (clone 124, for more details, please see our recent review [37]). Choi et al. [36] also used more stringent criteria for BCL2 positivity which resulted in low proportion of BCL2-positive cases (9.6 %).

The advantage of BCL2 protein expression as a predictive marker is its easy detection. Routine immunohistochemical analyses of BCL2 expression in FFPE tissue samples could easily be performed at the time of diagnosis in any pathology laboratory with standard facilities.

High EGFR expression was associated with poor BCSS in the present series, which is consistent with the findings of Viale et al. [19]. Such patients may benefit from anti-EGFR-targeted therapies and should be included in relevant clinical trials.

The proportion of EGFR gene amplification observed in this work (7.5 %) was similar to that reported by Shah (5 %) [3]. The EGFR-positive tumors identified in both studies exhibited high levels of amplification; however, the low frequency of EGFR amplification probably precluded any significant association with outcomes in TNBC.

We observed a trend to worse survival among patients with CMYC gene amplification, which is consistent with previously published results for an unselected cohort of BC patients [18]. CMYC protein expression was found in the majority of the TNBC cohort examined in this work. A recent study demonstrated that xenografts with elevated MYC protein expression regressed following treatment with cyclin-dependent kinase inhibitors [38], suggesting that clinical investigations using these drugs may reveal new treatment possibilities for this particular subgroup of TNBC patients.

TOP2A amplification is also a potential predictor of responsiveness to anthracycline-based therapy [18, 21, 22, 39, 40]. However, TOP2A amplification is rare in TNBC, occurring in only 2.1 % of our anthracycline-treated cohort when detected based on gene/chromosome ratios or 5.6 % when

detected based on the TOP2A gene copy number. The low frequency of TOP2A amplification probably precluded the identification of any association with outcome in this work. A study comparing cyclophosphamide, epirubicin, and 5-fluorouracil (CEF) and cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) treatments in unselected BC patients demonstrated that both TOP2A amplification and TOP2A deletion have important effects on outcome [39–41]. The anthracycline-containing CEF regimen was found to be superior to the CMF treatment for all tumors with TOP2A aberrations (including both TOP2A amplifications and deletions). This phenomenon has not yet been fully explained. However, TOP2A deletion may be a surrogate marker for chromosomal and genomic instability, which would explain the sensitivity of TOP2A-deleted tumors to the DNA-damaging anthracyclines [42]. The roles of TOP2A amplification and deletion as predictors should be analyzed in a larger prospective study.

A recent study identified TOP2A protein overexpression as a predictor of pCR based on a multivariate analysis [15]. However, our analysis of TOP2A protein expression (based on a histoscore threshold of >190) revealed no association with survival in adjuvant settings. This may be due to the intratumoral heterogeneity of TOP2A expression or variation in its expression during the cell cycle. As such, further investigation is required to determine the utility of TOP2A protein expression as a predictor of outcomes in TNBC patients treated with anthracycline-based chemotherapies. Similarly, we found no association between Ki-67 expression and survival. Munzone et al. reported that Ki-67 labeling in excess of 35 % was associated with negative survival in node-negative TNBC [25]. On the other hand, others [43–45] have reported an association between strong Ki-67 labeling and pCR in unselected BC patients undergoing preoperative chemotherapy involving mitoxantrone with methotrexate; CMF; epirubicin/docetaxel; or 5-fluorouracil, epirubicin, and cyclophosphamide. However, many of these studies used different Ki-67 labeling thresholds and the predictive capacity of Ki-67 labeling has yet to be clinically validated.

A so-called anthracycline-based score (A-score) has been developed by analyzing gene expression in breast cancers and was shown to predict anthracycline responsiveness and resistance [46]. The score is calculated by considering three gene expression signatures: a TOP2A gene signature and two signatures relating to tumor invasion and immune response. Calculation of the A-score thus requires the extraction of mRNA from the tumor and microarray analysis of gene expression, which is not possible in all pathology labs. Moreover, the method is expensive to perform and requires the availability of a specialized team. Finally, it is not an in situ method and has not been validated for use with adjuvant therapies in TNBC. Conversely, BCL2 protein expression analysis is a simple and inexpensive in situ method.

Conclusion and further directions

To our best knowledge, this is the first study showing BCL2 protein expression as an independent predictor of outcome in basal-like (BL) TNBC treated with adjuvant anthracycline-based chemotherapy. If validated in prospective study, BCL2 protein expression could improve decision making on adjuvant chemotherapy in clinical practice [47, 48]. In addition, in vitro or in vivo studies using TNBC cell lines or xenografts should be conducted to provide mechanistic insights into the predictive role of BCL2 expression analysis.

Conflicts of interest MH and RT are owners of Intellmed stock.

Authors' contributions KB conceived, designed, and directed the study; analyzed the data; and wrote the manuscript. MS obtained clinical data. GK performed IHC (BCL2, TOP2A, EGFR, CMYC), and JV and LR performed the statistical analyses. JB had oversight of the study's progression and contributed to the data analysis and the writing of the manuscript. RT and VK performed the FISH experiments. KC and MH contributed to the editing of the manuscript. ZK contributed to the writing of the manuscript. All authors approved the manuscript.

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