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Original article

Prognostic, predictive abilities and concordance of BCL2 and TP53 protein expression in primary breast cancers and axillary lymph-nodes: A retrospective analysis of the Belgian three arm study evaluating anthracycline vs CMF adjuvant chemotherapy

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ABSTRACT

Given recent data on genetic heterogeneity within and individual's tumor, we investigated if there were differences in the prognostic and predictive abilities of BCL2 and TP53 protein expression in primary breast cancer (TU) and corresponding axillary lymph-nodes (LN). We used patient samples from the adjuvant Belgian three-arm study which randomized between anthracycline containing regimens and traditional CMF. The endpoints analyzed were overall survival (OS), event-free survival (EFS) and interactions between chemotherapy regimens.

At a median follow-up of 15.6 years, BCL2 and TP53 (in both TU and LN) were significantly associated with OS but only in the first 5 years. Likewise, BCL2 and TP53 (in both TU and LN) were associated with EFS in the first 2 years after randomization, with no association after 2 years. BCL2 and TP53 remained statistically significant after adjustment for the standard clinical–pathological characteristics in regard to OS and EFS in the respective first years after randomization, (p value < 0.001 for both markers).

Furthermore, an interaction was found between high BCL2 expression in the TU (but not in LN) and benefit to CMF over anthracycline-based chemotherapy (interaction p value EFS: 0.042; OS = 0.01). No interaction was found for TP53 expression neither in TU nor in LN.

We conclude that BCL2 and TP53 were predictive biomarkers for better and worse survival respectively, but only in the first two to five years after diagnosis. BCL2 expression in the TU but not in the LN was predictive of increased benefit to CMF vs anthracycline-based chemotherapy.

List of abbreviations: BC, breast cancer; BCL2, B-cell leucemia/lymphoma 2; CMF, cyclophosphamide, methotrexate, fluorouracil regimen; EC, epirubicin, cyclophosphamide regimen; EFS, event free survival; ER, estrogen receptor; HEC, high epirubicin cyclophosphamide regimen; HER2, human epidermal growth factor receptor 2; LN, lymph nodes; OS, overall survival; PgR, progesteron receptor; TP53, tumor protein 53; TU, primary tumor.

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Introduction

The current available prognostic classifiers in the breast cancer are based on clinical and pathological factors and have shown to be useful estimating an individual risk of breast cancer relapse [1,2]. At the present, however there is a lack of robustly validated biomarkers that can predict for increased chance of benefit to standard cytotoxic chemotherapy.

BCL2 protein is a member of the BCL2 family of apoptosis targeting molecules acting as a powerful inhibitor of cell death [3]. It has been shown that BCL2 has strong independent prognostic abilities, independent of the Nottingham Prognostic Index [4] and increased expression is associated with an improved survival in breast cancer [5,6]. It is also strongly correlated with ER status [7]. Wild-type TP53 protein activates apoptosis, cell cycle arrest and senescence in response to DNA damage while alterations of TP53 cause loss of these functions. High TP53 expression is generally associated with a poor outcome in breast cancer [8], though TP53 expression is not highly correlated with mutation status as the most common TP53 mutation does not alter protein levels [9,10]. Many retrospective studies have attempted to associate the BCL2 and TP53 with chemotherapy benefit [10,11]. However, a biomarker's predictive ability can only be truly evaluated in the context of a randomized clinical trial.

Recent analysis using next generation sequencing technologies by Gerlinger and colleagues performed on multiple samples obtained from primary renal cell carcinomas and associated metastatic sites showed that no two samples were genetically identical [12]. They reported that about two-thirds of the mutations present in a single biopsy were not present in biopsies taken from across the same tumor. This and other data raise the possibility that presentation of a tumor's molecular profile obtained from single biopsy sample may not be adequate. It is unclear if these changes in receptor expression are a true biological phenomenon or may result from technical variables [13].

In the current study we aimed to assess the heterogeneity between primary breast cancers (TU) and the ipsilateral axillary lymph nodes (LN) status and to explore the concordance of BCL2 and TP53 protein expression. We did this by using samples from the Belgian three arm adjuvant trial which randomized 777 node positive women to higher dose epirubicin (HEC) vs lower dose epirubicin (EC) regimen vs traditional oral CMF (cyclophosphamide, methotrexate, fluorouracil) [14,15]. We next wanted to confirm the prognostic value of these biomarkers in a study with long-follow up. The randomization in this trial also allowed us to evaluate if BCL2 or TP53 were predictive of benefit to an anthracycline vs no anthracycline-based chemotherapy regimen (CMF) in early BC patients. We further evaluated if there were differences in these effects between TU and LN expression of these markers.

Patients and methods

Further information is available in the Supplementary Methods.

Patient population

Among total of 777 patients enrolled in phase III, randomized, multicenter study, conducted in Belgium and Luxembourg from 1988 to 1996 [14], 286 (37%) tumor samples were not available for biomarker analysis (Supplementary Table 1). The rest of 491 patients (63%) had evaluable tumor samples for biomarker analysis [14]. The original study protocol was reviewed and approved by the ethics committee of each participating institution in Belgium and all patients had to have signed informed consent at that time.

Three treatment regimens were used in this study. These were: **classic CMF** for six cycles (oral cyclophosphamide 100 mg/m^2 on days 1 through 14, methotrexate 40 mg/m^2 intravenously [IV] and fluorouracil 600 mg/m^2 IV on days 1 and 8 every 28 day); **low dose epirubicin (LEC)** for eight cycles (epirubicin 60 mg/m^2 IV and cyclophosphamide 500 mg/m^2 IV on day 1 every 21 days) or **high dose of epirubicin (HEC)** for eight cycles (epirubicin 100 mg/m^2 IV and cyclophosphamide 830 mg/m^2 IV on day 1 every 21 days). Fifteen year efficacy results which reported that the higher dose of epirubicin (HEC) was superior to a lower dose of epirubicin (LEC), but was equivalent to the CMF regiment, have been previously published [15].

Biomarker immunostaining and scoring

The BCL2 and TP53 central pathology analysis using immunohistochemistry (IHC) in TU and LN has been previously described [16]. Scoring was based on the estimated proportion of tumor cells staining positively: nuclear staining for TP53 and cytoplasmic staining for BCL2. A semiquantitative scoring system was used to evaluate focality staining (none: 0; 1: ≤25% of positive cells; 2: ≤50%; 3: ≤75%, and 4: ≤100%) and intensity staining (none: 0, weak: 1, intermediate: 2, and strong: 3) for both markers [16]. Cut-off for staining for TP53 and BCL2 was analyzed as dichotomous variables <25% and ≥25% positive staining cells, as previously pre-specified [17].

Statistical analyses

Expression of BCL2 and TP53 as dichotomous variables in both TU and LN were analyzed in relation to overall survival (OS) and event free survival (EFS) in all patients with available biomarker data and in regards to chemotherapy regimens interaction between CMF vs HEC (non-anthracycline vs anthracycline arm). Kaplan–Meier curves were drawn for EFS and OS. Cox proportional hazards regression analysis was used to estimate hazard ratios and 95% confidence intervals. Log-rank tests were performed for time-to-events end points.

The proportional hazards assumptions had been thoroughly investigated by plots of log (cumulative hazard function) vs time, partitioning the time axis and fitting models separately to each time interval (piecewise model), including time by covariate interaction terms in the model, assessing plots of Schoenfeld's

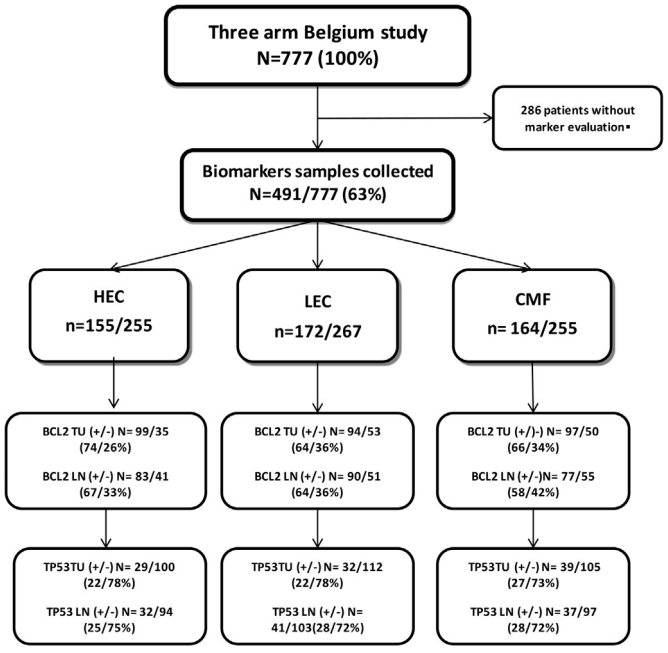

Fig. 1. Flow diagram of the breast cancer specimens used for the study.

Table 1
Association of BCL2 and TP53 with patient's and tumor's characteristics.

	BCL2						TP53					
	Tumor (TU)			Lymph node (LN)			Tumor (TU)			Lymph node (LN)		
	All BCL2+/BCL2−	BCL2 positive cases		All BCL2+/BCL2−	BCL2 positive cases		All TP53+/TP53−	TP53 positive cases		All P53+/p53−	TP53 positive cases	
	$N = 428$ (+/−) = 290/138	BCL2 TU+ (%)	p Value[a]	$N = 397$ (+/−) = 215/182	BCL2 LN+ (%)	p Value[a]	$N = 417$ (+/−) = 100/317	TP53TU+ (%)	p Value[a]	$N = 404$ (+/−) = 110/294	TP53 LN+ (%)	p Value[a]
Age												
<50 years	234	161 (69%)	0.61	223	139 (62%)	0.76	230	54 (23%)	0.79	226	60 (27%)	0.73
≥50 years	194	129 (66%)		174	111 (64%)		187	46 (25%)		178	50 (28%)	
Menopausal status												
Premenopausal	258	179 (69%)	0.38	246	154 (63%)	0.88	253	61 (24%)	0.97	250	65 (26%)	0.55
Postmenopausal	170	111 (65%)		150	95 (63%)		163	39 (24%)		153	44 (29%)	
Histotype												
Ductal	345	229 (66%)	0.09	324	198 (61%)	0.09	334	85 (25%)	0.13	331	94 (28%)	0.15
Lobular	51	40 (78%)		43	32 (74%)		51	8 (16%)		44	8 (18%)	
Ductal and lobular	21	15 (71%)		19	15 (79%)		21	5 (24%)		18	5 (28%)	
Other	6	4 (67%)		6	3 (50%)		6	1 (17%)		6	2 (33%)	
pT size%												
≤2 cm	199	154 (77%)	<0.001[b]	169	121 (72%)	0.01[b]	194	42 (22%)	0.12[b]	172	45 (26%)	0.40[b]
2–5 cm	173	104 (60%)		168	99 (59%)		170	47 (28%)		170	50 (29%)	
>5 cm	5	5 (71%)		9	5 (56%)		8	4 (50%)		9	4 (44%)	
Histologic grade												
1	77	62 (81%)	<0.001	69	54 (78%)	<0.001	78	5 (6%)	<0.001	70	7 (10%)	<0.001
2	208	147 (71%)		190	128 (67%)		198	50 (25%)		194	54 (28%)	
3	90	43 (48%)		90	36 (40%)		88	35 (40%)		92	39 (42%)	
N° of positive LN												
1–3	252	181 (72%)	0.02	217	141 (65%)	0.12	245	51 (21%)	0.03	223	58 (26%)	0.48
4–9	120	77 (64%)		120	78 (65%)		114	29 (25%)		122	34 (28%)	
≥10	56	32 (57%)		60	31 (52%)		58	20 (34%)		59	18 (31%)	
ER status												
Positive	254	197 (78%)	<0.001	233	172 (74%)	<0.001	246	47 (19%)	<0.001	234	52 (22%)	<0.001
Negative	131	70 (53%)		125	58 (46%)		129	47 (36%)		129	52 (40%)	
PgR status												
Positive	238	186 (78%)	<0.001	209	156 (75%)	<0.001	232	43 (19%)	<0.001	214	49 (23%)	0.003
Negative	146	79 (54%)		148	72 (49%)		142	51 (36%)		148	55 (37%)	
HER2/neu[c]												
Positive	47	20 (43%)	<0.001	47	20 (43%)	<0.001	45	22 (49%)	<0.001	45	23 (51%)	<0.001
Negative	210	170 (81%)		173	132 (76%)		212	38 (18%)		183	36 (20%)	
Events (EFS)	251	168 (67%)	–	246	151 (61%)	–	244	63 (26%)	–	248	74 (30%)	–
Deaths (OS)	170	106 (62%)	–	170	97 (57%)	–	163	48 (29%)	–	170	57 (34%)	–

[a] p Value = chi-square (Mantel–Haenszel chi-square for the variables grade and n° of positive lymph nodes) – association between BCL2 or TP53 TU or LN and clinico-pathological factors.
[b] ≤2 cm vs larger than 2 cm.
[c] HER2/neu status was available in only half of the patients: 257/491 = 52%.

Table 2A
Univariate analysis of BCL2 and TP53 (TU and LN): overall survival, considering the follow-up intervals till 5 years, >5–10 years and >10 years.

	Overall survival (OS)					
	Till 5 years follow-up		>5–10 years follow-up		>10 years follow-up	
	HR (95% CI)	*p* Value	HR (95% CI)	*p* Value	HR (95% CI)	*p* Value
BCL2 TU status positive vs negative	0.43 (0.27–0.67)	<0.001	1.06 (0.61–1.85)	0.84	1.22 (0.57–2.60)	0.62
BCL2 LN status positive vs negative	0.39 (0.25–0.60)	<0.001	1.16 (0.66–2.04)	0.61	1.15 (0.54–2.44)	0.72
TP53 TU status positive vs negative	2.27 (1.42–3.63)	<0.001	1.00 (0.53–1.89)	1	1.05 (0.45–2.42)	0.91
TP53 LN status positive vs negative	2.52 (1.63–3.91)	<0.001	1.17 (0.65–2.10)	0.60	0.61 (0.24–1.60)	0.32

Table 2B
Univariate analysis of BCL2 and TP53 (TU and LN): event-free survival, considering the follow-up intervals till 2 years, >2–10 years and >10 years.

	Event-free survival (EFS)					
	Till 2 years follow-up		>2–10 years follow-up		>10 years follow-up	
	HR (95% CI)	*p* Value	HR (95% CI)	*p* Value	HR (95% CI)	*p* Value
BCL2 TU status positive vs negative	0.55 (0.32–0.95)	0.03	0.93 (0.65–1.32)	0.69	1.29 (0.70–2.36)	0.42
BCL2 LN status positive vs negative	0.51 (0.30–0.85)	0.009	1.01 (0.71–1.45)	0.95	1.07 (0.61–1.88)	0.82
TP53 TU status positive vs negative	2.40 (1.39–4.14)	0.002	1.05 (0.71–1.57)	0.79	0.87 (0.43–1.72)	0.68
TP53 LN status positive vs negative	2.76 (1.65–4.62)	<0.001	1.03 (0.70–1.52)	0.88	1.02 (0.54–1.90)	0.96

residuals against time and applying the supremum test for proportional hazards assumption (martingale-based residuals).

To assess whether the BCL2 status (TU, LN) and TP53 status (TU, LN) were independently related to OS and EFS, a first multivariate Cox's proportional hazards model including as usual clinical–pathological covariates was fitted to the data. In a second step, BCL2 (TU, LN) and TP53 (TU, LN) were added to the first model and their significance was assessed using the change in the likelihood ratio χ^2 value, stratified by chemotherapy arm.

To test whether treatment effects (CMF, HEC) were homogeneous across the stratification factors (BCL2 status, TP53 status), subgroup analyses of OS and EFS with the use of Cox's proportional hazards model with interaction terms were included. Forest plots were used to summarize the results of the subgroup analyses. Median follow-up time was estimated based on the reverse Kaplan–Meier estimator [18]. Interaction tests were not adjusted for estrogen receptor (ER) status and menopausal status due to small number of patients in each group.

The χ^2 test and Fisher's exact test were used to test the statistical significance of differences in discrete data; student's *t*-test and Mann–Whitney's test were used to test the statistical significance of differences in continuous data. To assess the concordance between tumor and lymph node assessment, the Kappa statistic was used. All reported *p*-values are two-sided. *p*-Values < 0.05 were considered to indicate statistical significance. Statistical analyses were done using SAS version 9.3 (SAS Institute Inc., Cary, NC, USA).

Table 2C
Univariate analysis of the usual clinical–pathological characteristics: overall survival till 5 years follow-up.

Factor	Overall survival (OS)	
	Till 5 years follow-up	
	HR (95% CI)	*p* Value
Age ≥50 vs <50 years	1.25 (0.82–1.90)	0.30
Menopausal status Post vs Pre	1.10 (0.72–1.68)	0.67
Histologic type Ductal vs Lobular/other	1.63 (0.87–3.06)	0.13
Tumor size >2 vs ≤2 cm	1.56 (0.99–2.45)	0.05
Tumor grade 3 vs 2 vs 1	1.72 (1.23–2.41)	0.002
N° of positive LN ≥4 vs <4	2.33 (1.52–3.57)	<0.001
ER status positive vs negative	0.51 (0.33–0.80)	0.003
PgR status positive vs negative	0.51 (0.32–0.79)	0.003
HER2 status positive vs negative	1.59 (0.83–3.08)	0.16

Results

Biomarkers were assessed in 63% (491/777) of the original trial population (Fig. 1). Of these 491 patients, 155 treated with HEC, 172 treated with LEC and 164 treated with CMF. The median follow-up was 15.6 years (range 0.4–21.9 years). There was no difference between the patient and tumor characteristics amongst patients with (491/777; 63%) and without biomarker (286/777; 37%) assessment (Supplementary Table 1). There was no difference in OS ($p = 0.81$) and EFS ($p = 0.75$) between the two cohorts.

Biomarker concordance between tumor and lymph nodes

There was moderate to high concordance seen in samples that had both BCL2 and TP53 assessed in both primary tumor (TU) and axillary lymph nodes (LN) (324/491). For BCL2, the concordance was $\kappa = 0.71$ (95%CI: 0.62–0.79) and for TP53 was estimated a $\kappa = 0.84$ (95%CI: 0.77–0.90), both $p < 0.001$.

Table 2D
Univariate analysis of the usual clinical–pathological characteristics: event-free survival till 2 years follow-up.

Factor	Event-free survival (EFS)	
	Till 2 years follow-up	
	HR (95% CI)	*p* Value
Age ≥50 vs <50 years	1.23 (0.76–1.99)	0.41
Menopausal status Post vs Pre	1.19 (0.72–1.94)	0.50
Histologic type Ductal vs Lobular/other	1.63 (0.78–3.41)	0.20
Tumor size >2 vs ≤2 cm	1.33 (0.80–2.20)	0.27
Tumor grade 3 vs 2 vs 1	1.72 (1.17–2.53)	0.006
N° of positive LN ≥4 vs <4	2.36 (1.44–3.86)	<0.001
ER status positive vs negative	0.53 (0.32–0.88)	0.01
PgR status positive vs negative	0.64 (0.38–1.06)	0.08
HER2 status positive vs negative	3.02 (1.61–5.67)	<0.001

Association of BCL2 and TP53 with clinico-pathological characteristics

We found that high BCL2 expression (in both TU and LN) was significantly associated with favorable biological features such as tumor size ≤2 cm (TU $p < 0.001$, LN $p < 0.01$), low histological grade (TU $p < 0.001$, LN $p < 0.01$), positive ER and PgR receptors status (TU $p < 0.001$, LN $p < 0.01$), and absence of HER2 overexpression (TU $p < 0.001$, LN $p < 0.01$) (Table 1). In contrast and as expected, high TP53 expression (both TU and LN) was associated with high histologic grade (TU $p < 0.001$, LN $p < 0.01$), ER-negativity (TU $p < 0.001$, LN $p < 0.01$), and HER2 positivity (TU $p < 0.001$, LN $p < 0.01$). There was a significant association between BCL2 and TP53 expression in the primary tumor (TU) and the number of lymph node involvement (TU $p < 0.001$, LN $p < 0.01$ for both), but none for expression in the LN (Table 1).

Associations between BCL2 and TP53 expression (TU, LN) and prognosis (all patients pooled)

If we look at the entire follow-up period, there seemed to be an issue with the proportional hazards assumption for the BCL and TP53. Therefore, we considered intervals of the follow-up period. The proportional hazards assumption for OS was found to be fulfilled for the following follow-up time intervals: till 5 years, >5–10 years and >10 years. Likewise, for EFS, the proportional hazards assumption was met for the following follow-up time intervals: till 2 years, >2–10 years and >10 years.

BCL2 and TP53 were significantly related to OS in the first 5 years after randomization: BCL2 TU positive: HR 0.43 (0.27–0.67), BCL2 LN positive: HR 0.39 (0.25–0.60), TP53 TU positive: 2.27 (1.42–3.63), TP53 LN positive: 2.52 (1.63–3.91) (Table 2A). However, after 5 years from randomization this OS difference was no longer significant, suggesting a time dependent effect (Table 2A).

BCL2 and TP53 were significantly related to EFS in the first 2 years after randomization: BCL2 TU positive 0.55 (0.32–0.95), BCL2 LN positive 0.51 (0.30–0.85), TP53 positive TU 2.40 (1.39–4.14) and TP53 positive LN 2.76 (1.65–4.62) (Table 2B). There was no statistical evidence for associations between the biomarkers and EFS after 2 years following the randomization (Table 2B). Multivariate analysis, which was made by adding biomarkers from TU and LN on basic model (Tables 2C and 2D) comprised with standard prognostic parameters, did confirm these findings (Tables 2E and 2F).

Table 2E
Multivariate analysis of the usual clinical–pathological characteristics and BCL2 (TU, LN) and TP53 (TU, LN): overall survival till 5 years follow-up.

	Overall survival (OS)	
	Till 5 years follow-up	
	−2 Log likelihood	*p* Value (compared to the basic model)
Basic model: Tumor grade (3 vs 2 vs 1) N° of positive LN (≥4 vs <4) ER status (positive vs negative)	649.566	
Adding BCL2 TU status (positive vs negative)	546.319	<0.001
Adding BCL2 LN status (positive vs negative)	571.798	<0.001
Adding TP53 TU status (positive vs negative)	530.534	<0.001
Adding TP53 LN status (positive vs negative)	586.353	<0.001

Associations between BCL2 (TU, LN) and TP53 (TU, LN) expression and chemotherapy benefit

The next aim was to determine if the effect of the anthracycline-containing chemotherapy was different to that of CMF regimen according to biomarker status in the TU and LN. As the lower dose arm (EC) was inferior to the higher dose epirubicin arm (HEC), this arm was not included in these analyses (i.e. HEC was compared with CMF). As seen in Fig. 2(A–D), there was evidence of a heterogeneous effect between BCL2 expression in the TU, OS (interaction p value = 0.01) and EFS (interaction p value = 0.042) between CT regimens. In other words, high expression of BCL2 in the TU was associated with benefit from CMF over HEC chemotherapy (Fig. 3). However, this interaction was not significant for BCL2 expression in the lymph nodes, though the direction was the same.

There was no evidence of interaction between TP53 status (TU or LN) and chemotherapy regimen (Fig. 2E–H).

Discussion

In this study, using samples from a randomized adjuvant clinical trial evaluating anthracycline vs non-anthracycline chemotherapy regimens, we report three main findings. First, we found that prognostic value of BCL2 and TP53 expression in early BC patients with over 15 years follow-up, was significant for OS and EFS only in the first five years and two years of follow-up period, respectively.

Table 2F
Multivariate analysis of the usual clinical–pathological characteristics and bcl2 (TU, LN) and TP53 (TU, LN): event-free survival till 2 years follow-up.

	Event-free survival (EFS)	
	Till 2 years follow-up	
	−2 Log likelihood	*p* Value (compared to the basic model)
Basic model: Tumor grade (3 vs 2 vs 1) N° of positive LN (≥4 vs <4) ER status (positive vs negative) Her2 status (positive vs negative)	263.119	
Adding BCL2 TU status (positive vs negative)	214.456	<0.001
Adding BCL2 LN status (positive vs negative)	235.689	<0.001
Adding TP53 TU status (positive vs negative)	224.446	<0.001
Adding TP53 LN status (positive vs negative)	221.702	<0.001

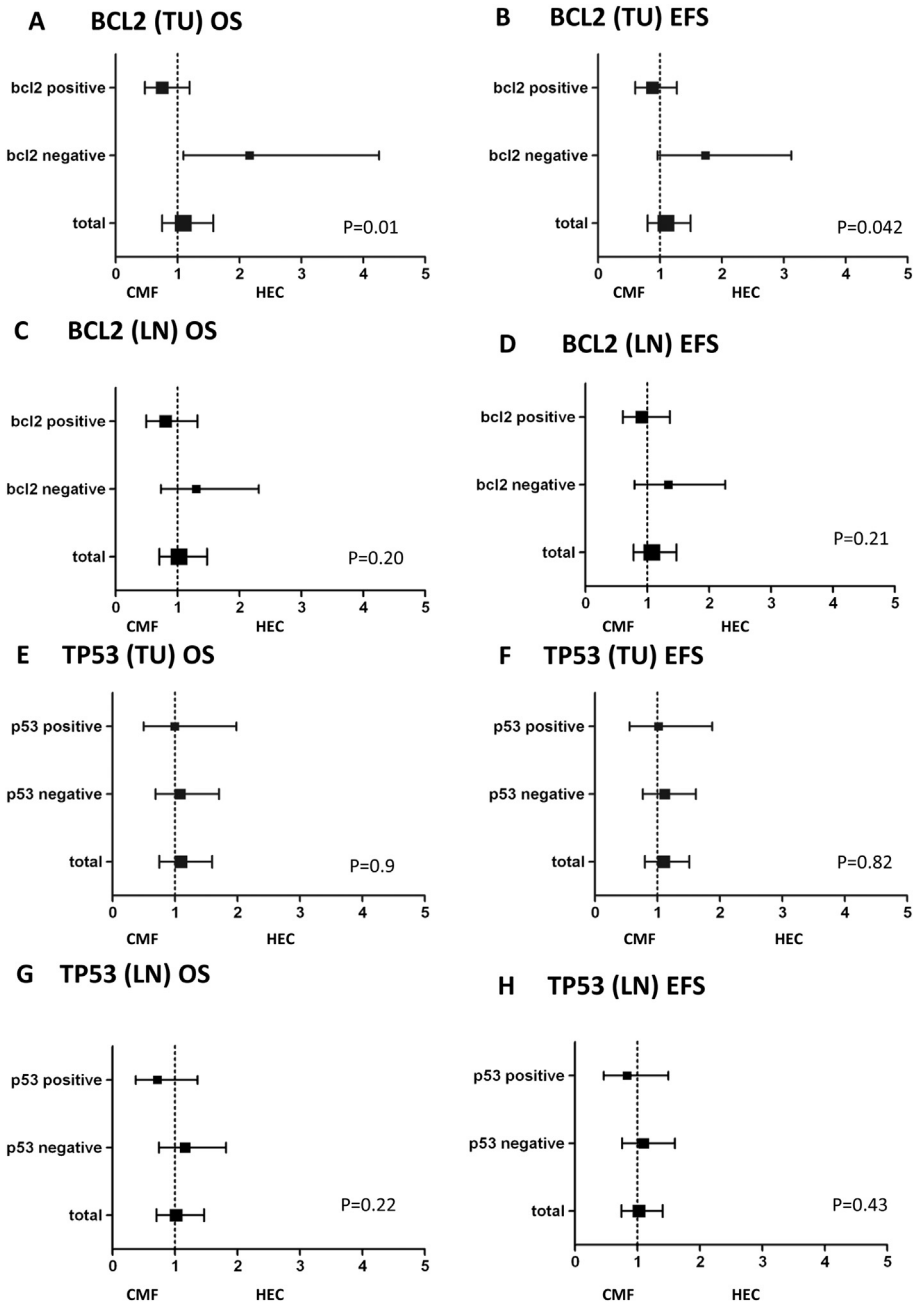

Fig. 2. Forest plots for the interaction test according to different BCl2 and TP53 expression in primary tumor (TU) and ipsilateral axillary lymph nodes (LN) and response on chemotherapy (CMF vs HEC) presented for OS and EFS. A. BCL2 (TU) OS; B. BCL2 (TU) EFS; C. BCL2 (LN) OS; D. BCL2 (LN) EFS; E. TP53 (TU) OS; F. TP53 (TU) EFS; G. TP53 (LN) OS; H. TP53 (LN) EFS.

Secondly, we found that high BCL2 expression in the TU but not in the LN was significantly associated with a higher likelihood of benefit to CMF over HEC. Finally, we note that the concordance of these biomarkers between primary TU and LN was not perfect however, we did not find differences in their prognostic abilities based on anatomical localization.

Our results concerning BCL2 expression and prognosis confirm previous literature reports. BCL2 is often associated with ER expression and an indolent breast cancer phenotype of low grade [5,6,19]. A prospective trial with more than 11,000 early BC patients demonstrated robust prognostic significance of BCL2 protein expression, which was independent of ER and other tumor characteristics and was present across all molecular subtypes (ER +/−, HER2 +/− and triple negative) [6]. In contrast to our data, BCL2 positivity in the mentioned study continued to be associated with a favorable prognostic effect throughout the whole follow-up period of 8.4 years. Of note, BCL2 is part of the 21-gene signatures (Onco*type* DX® Genomic Health), though BCL2 protein expression and transcriptional levels are not perfectly correlated [6,20].

Most studies looking at the association between TP53 mutation and survival had found a poorer prognosis for breast cancer patients with increased TP53 expression [10,21–23]. Such findings have been confirmed in a meta-analysis with 16 studies for both node-negative and node-positive breast cancer patients [8]. We found that there was no differential effect between TP53 expression and chemotherapy response supporting existing data in the literature [23].

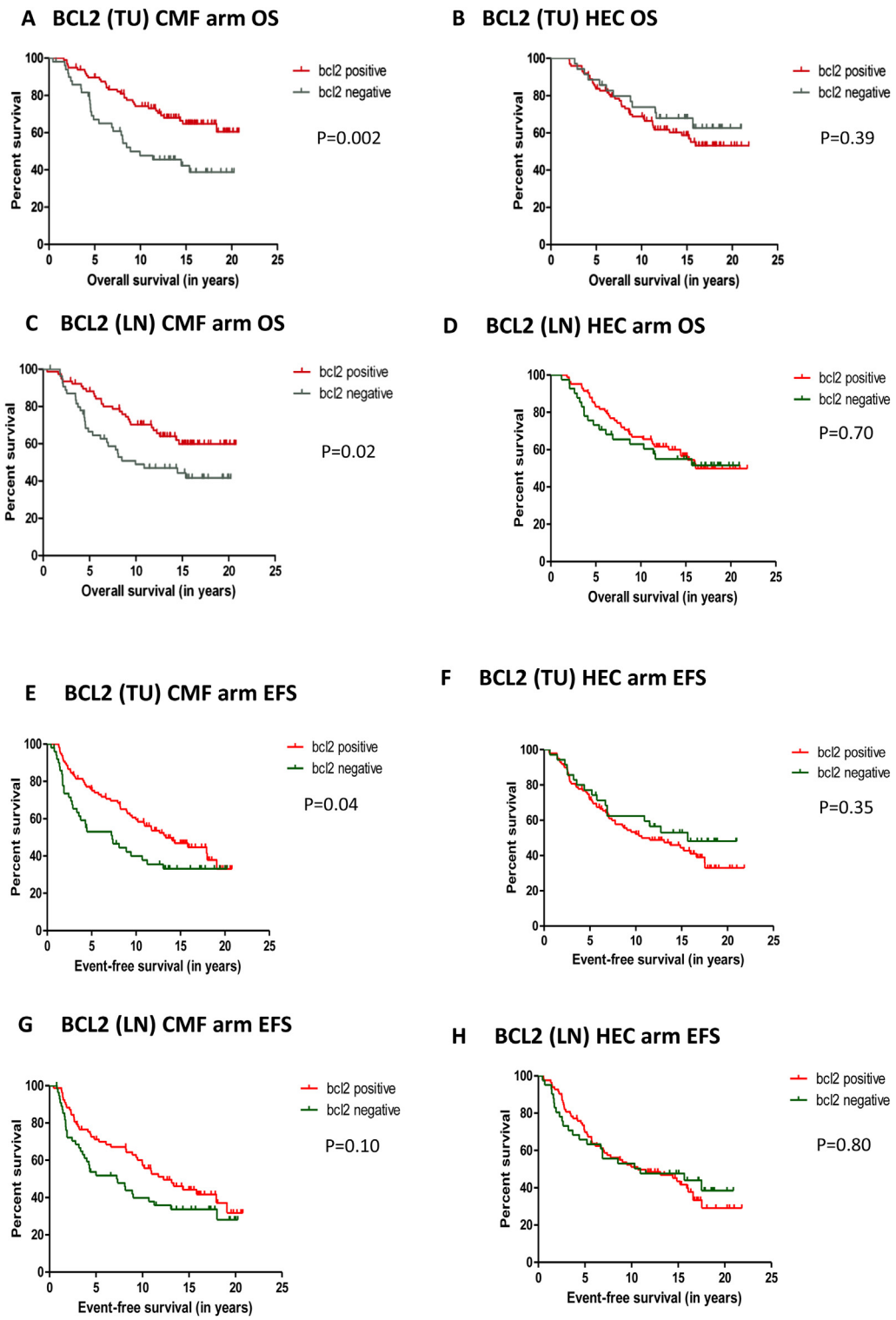

Fig. 3. Kaplan–Meier survival curves (OS and EFS) according to different BCL2 status in primary tumor (TU) and ipsilateral axillary lymph nodes (LN) and response on chemotherapy: A, C, E, G – response on CMF and B, D, F, H – response on HEC. A. BCL2 (TU) CMF arm OS; B. BCL2 (TU) HEC OS; C. BCL2 (LN) CMF arm OS; D. BCL2 (LN) HEC arm OS; E. BCL2 (TU) CMF arm EFS; F. BCL2 (TU) HEC arm EFS; G. BCL2 (LN) CMF arm EFS; H. BCL2 (LN) HEC arm EFS.

In this study, taking advantage of the randomization, we also show that high BCL2 expression in the primary TU but not in the LN was associated with benefit from CMF over anthraycline-based adjuvant chemotherapy. While this did not reach statistical significance for LN expression, we also observed the same trend (Fig. 3). Other non-randomized clinical studies have suggested that BCL2 expression is associated with benefit from CMF and tamoxifen adjuvant therapy [24,25]. This effect has been attributed to the fact that BCL2 is an estrogen responsive gene [17,24,25]. As we could not confidently adjust the interaction test

for the ER status and menopausal status due to the small number of cases, further confirmation of a CMF benefit will be required. Given the fact that likelihood of amenorrhea following CMF chemotherapy is high, especially in older premenopausal women, it might be that effect seen in BCL2 positive patients, which are often ER-positive and endocrine sensitive, is the consequence of the higher rate of CMF-induced amenorrhea in comparison with HEC regimen. Notable, in the time when this study was performed, tamoxifen was given solely to postmenopausal patients with ER positive or unknown receptor status. A potential clinical implication of this finding could be that clinicians could prescribe the less toxic CMF regimen to older patients who might have contraindications for or might not tolerate anthracycline-based regimens. Of note, better responses from anthracycline-based chemotherapy for patients with low vs high BCL2 expression have also been reported by another group using 485 retrospective samples from several non-randomized series, though the exact cut-off used was not specified.

To the best of our knowledge, this is the first study that has investigated TP53 and BCL2 protein expression and their prognostic and predictive associations in both primary TU and LN.

However, our study has some limitations. First, the reliability of the IHC assay is affected by several factors of variability, both analytical and pre-analytical that may influence the final results. In addition, since no international standards yet exist, the definitions of optimal markers' cut-off for positivity is unknown. Hence, levels used to score positive tumors by IHC in our study, whilst pre-specified [16], were arbitrarily chosen. Second, this was a retrospective analysis with multiple analyses undertaken for hypothesis generation. Hence, the differences that we found between TU and LN expression should be further validated, especially given the many technical issues regarding the incomplete number of samples analyses (compared to the original trial cohort), IHC analyses and interpretation. Third, we are aware of the fact that IHC method assesses only the presence or absence of a protein, without necessarily providing information regarding the form or function of that protein. IHC does not seem to be the best method for the TP53 status determination since TP53 protein expression does not correlate with TP53 gene mutation and may result in a certain percentage of false positive and false negative results [9,27]. However, the IHC widely available, is able to be performed on stored FFPE tumor samples and is low cost.

Conclusion

To the best to our knowledge this study is the first to evaluate level of concordance of BCL2 and TP53 between primary breast cancer and ipsilateral axillary lymph nodes, and their prognostic impact in patients treated with antracycline-based vs CMF chemotherapy for early breast cancer. We found that both biomarkers has significant impact on survival only in the period of first 5 years of follow-up. For the first time we show that BCL2 expression may suggest differential benefit to CMF vs anthracycline adjuvant chemotherapy in a randomized clinical trial dataset, although this finding needs to be further validated.

Author contributions

No writing assistance was used for this manuscript. IBS, EA, LA, conceived the study; IBS, LA, EA, and SL analyzed and interpreted the data; AdL, and DL contributed to the sample processing and BCL2, TP53 testing; SD coordinated collection of the samples of the study; all authors contributed to the writing of the manuscript and approved the final version for submission.

Conflicts of interest statement

All authors have declared there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.breast.2014.03.012.

References

[1] Haybittle JL, Blamey RW, Elston CW, et al. A prognostic index in primary breast cancer. Br J Cancer 1982;45:361–6.
[2] Ravdin PM, Siminoff LA, Davis GJ, et al. Computer program to assist in making decisions about adjuvant therapy for women with early breast cancer. J Clin Oncol 2001;19:980–91.
[3] Evan GI, Brown L, Whyte M, et al. Apoptosis and the cell cycle. Curr Opin Cell Biol 1995;7:825–34.
[4] Callagy GM, Pharoah PD, Pinder SE, et al. Bcl-2 is a prognostic marker in breast cancer independently of the Nottingham Prognostic Index. Clin Cancer Res 2006;12:2468–75.
[5] Callagy GM, Webber MJ, Pharoah PD, et al. Meta-analysis confirms BCL2 is an independent prognostic marker in breast cancer. BMC Cancer 2008;8:153.
[6] Dawson SJ, Makretsov N, Blows FM, et al. BCL2 in breast cancer: a favourable prognostic marker across molecular subtypes and independent of adjuvant therapy received. Br J Cancer;103:668–75.
[7] Silvestrini R, Veneroni S, Daidone MG, et al. The Bcl-2 protein: a prognostic indicator strongly related to p53 protein in lymph node-negative breast cancer patients. J Natl Cancer Inst 1994;86:499–504.
[8] Pharoah PD, Day NE, Caldas C. Somatic mutations in the p53 gene and prognosis in breast cancer: a meta-analysis. Br J Cancer 1999;80:1968–73.
[9] Di Leo A, Tanner M, Desmedt C, et al. p-53 gene mutations as a predictive marker in a population of advanced breast cancer patients randomly treated with doxorubicin or docetaxel in the context of a phase III clinical trial. Ann Oncol 2007;18:997–1003.
[10] Fernandez-Cuesta L, Oakman C, Falagan-Lotsch P, et al. Prognostic and predictive value of TP53 mutations in node-positive breast cancer patients treated with anthracycline- or anthracycline/taxane based adjuvant therapy: results from the BIG 02-98 phase III trial. Breast Cancer Res;14:R70.
[11] Mottolese M, Benevolo M, Del Monte G, et al. Role of P53 and BCL-2 in high-risk breast cancer patients treated with adjuvant anthracycline-based chemotherapy. J Cancer Res Clin Oncol 2000;126:722–9.
[12] Gerlinger M, Rowan AJ, Horswell S, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N Engl J Med;366:883–92.
[13] Lindstrom LS, Karlsson E, Wilking UM, et al. Clinically used breast cancer markers such as estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 are unstable throughout tumor progression. J Clin Oncol;30:2601–8.
[14] Piccart MJ, Di Leo A, Beauduin M, et al. Phase III trial comparing two dose levels of epirubicin combined with cyclophosphamide with cyclophosphamide, methotrexate, and fluorouracil in node-positive breast cancer. J Clin Oncol 2001;19:3103–10.
[15] de Azambuja E, Paesmans M, Beauduin M, et al. Long-term benefit of high-dose epirubicin in adjuvant chemotherapy for node-positive breast cancer: 15-year efficacy results of the Belgian multicentre study. J Clin Oncol 2009;27:720–5.
[16] Cardoso F, Di Leo A, Larsimont D, et al. Evaluation of HER2, p53, bcl-2, topoisomerase II-alpha, heat shock proteins 27 and 70 in primary breast cancer and metastatic ipsilateral axillary lymph nodes. Ann Oncol 2001;12:615–20.
[17] Cardoso F, Paesmans M, Larsimont D, et al. Potential predictive value of Bcl-2 for response to tamoxifen in the adjuvant setting of node-positive breast cancer. Clin Breast Cancer 2004;5:364–9.
[18] Schemper M, Smith TL. A note on quantifying follow-up in studies of failure time. Control Clin Trials 1996;17:343–6.
[19] Daidone MG, Luisi A, Veneroni S, et al. Clinical studies of Bcl-2 and treatment benefit in breast cancer patients. Endocr Relat Cancer 1999;6:61–8.
[20] Sparano JA. TAILORx: trial assigning individualized options for treatment (Rx). Clin Breast Cancer 2006;7:347–50.

[21] Silvestrini R, Benini E, Daidone MG, et al. p53 as an independent prognostic marker in lymph node-negative breast cancer patients. J Natl Cancer Inst 1993;85:965–70.
[22] Allred DC, Clark GM, Elledge R, et al. Association of p53 protein expression with tumor cell proliferation rate and clinical outcome in node-negative breast cancer. J Natl Cancer Inst 1993;85:200–6.
[23] Bonnefoi H, Piccart M, Bogaerts J, et al. TP53 status for prediction of sensitivity to taxane versus non-taxane neoadjuvant chemotherapy in breast cancer (EORTC 10994/BIG 1-00): a randomised phase 3 trial. Lancet Oncol;12:527–39.
[24] Gasparini G, Barbareschi M, Doglioni C, et al. Expression of bcl-2 protein predicts efficacy of adjuvant treatments in operable node-positive breast cancer. Clin Cancer Res 1995;1:189–98.
[25] Elledge RM, Green S, Howes L, et al. bcl-2, p53, and response to tamoxifen in estrogen receptor-positive metastatic breast cancer: a Southwest Oncology Group study. J Clin Oncol 1997;15:1916–22.
[27] Bertheau P, Turpin E, Rickman DS, et al. Exquisite sensitivity of TP53 mutant and basal breast cancers to a dose-dense epirubicin-cyclophosphamide regimen. PLoS Med 2007;4:e90.