original article

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Evaluation of the prognostic and predictive value of p53 and Bcl-2 in breast cancer patients participating in a randomized study with dose-dense sequential adjuvant chemotherapy

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Purpose: To assess the prognostic and predictive significance of p53 and Bcl-2 protein expression in high risk patients with breast cancer treated with dose-dense sequential chemotherapy.
Patients and methods: From June 1997 until November 2000, 595 patients were randomized to three cycles of epirubicin (E) 110 mg/m^2 followed by three cycles of paclitaxel (P) 250 mg/m^2 followed by three cycles of 'intensified' CMF (cyclophosphamide 840 mg/m^2, methotrexate 47 mg/m^2 and fluorouracil 840 mg/m^2) or to four cycles of E, followed by four cycles of CMF. p53 and Bcl-2 expression was investigated by immunohistochemistry in 392 and 397 patients respectively.
Results: Positive expression of p53 was detected in 104 (26.5%) patients and was significantly associated with negative hormonal status, worse histologic grade, higher incidence of disease relapse and higher rate of death. p53 positive expression was a significant negative predictor of overall survival (OS) (P = 0.002) and disease-free survival (DFS) (P = 0.001). Negative expression of Bcl-2 was detected in 203 (51%) patients and was significantly associated with negative hormonal status. Multivariate analysis revealed that, positive p53 expression, higher number of positive nodes and worse tumor grade were related to significantly poorer OS and DFS.
Conclusions: For both treatments, p53 positive expression was a significant negative prognostic factor for OS and DFS while Bcl-2 was not. No predictive ability of p53 status or Bcl-2 status for paclitaxel treatment was evident.
Key words: breast cancer, adjuvant chemotherapy, p53, Bcl-2, paclitaxel

introduction

Randomized clinical trials have clearly demonstrated the efficacy of systemic adjuvant chemotherapy or hormonal therapy in women with breast cancer. However, the survival improvement is modest mainly due to the unselective inclusion of patients within a broad category of risk [1]. Therefore, the identification of biological markers that might have the ability to predict therapeutic response is crucial. Axillary lymph node status, tumor size and tumor grade are of prognostic value in patients with operable breast cancer, but only the expression of hormonal receptors is a clinically useful marker that predicts response to treatment [2–3]. Among the biological markers investigated, p53 and bcl-2 genes have received considerable attention as promising prognostic and predictive markers. These genes are involved in growth control and apoptosis pathways, which appear to play a key role in tumor progression and in response to anticancer agents [4–8].

The p53 gene encodes a nuclear phosphoprotein of 53 kDa that functions as a multifunctional transcription factor involved in the control of cell cycle, in repair after DNA damage, and in apoptosis [9]. p53 overexpression by immunohistochemistry (IHC) has been identified in 11–55% of invasive breast carcinomas. A large number of studies have assessed the prognostic value of p53 alterations yielding some conflicting

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results [7, 10–21]. Most of these studies demonstrate a worse outcome in case of p53 overexpression. p53 overexpression has been correlated with high histological grade, estrogen receptor negativity, shorter disease free survival (DFS) and overall survival (OS) [7, 10, 12–14, 15–17, 19, 21]. In contrast, other studies showed no association or trend towards a worse outcome, not reaching statistical significance [7, 11, 18, 20]. Lack of unanimity of results may be due to differences in technique, study design, or population, as well as the subjectivity inherent in some approaches [7].

The results on the role of p53 status as predictor of response to cytotoxic agents in the adjuvant or neoadjuvant setting are more contradictory (review article [7, 22–29]). The Cancer and Leukemia Group B (CALGB) 8541 randomized trial and a companion trial 8869 initially showed that the effect of dose intensification of doxorubicin on DFS and OS was not associated with p53 expression status in 397 node positive breast cancer patients [22]. In their updated analysis of the original 397 tumors and 595 additional tumors they showed that p53 interacted with dose in predicting DFS but not OS [23]. A study on 441 premenopausal node-negative breast cancer patients participating in the European Organization for Research and Treatment of Cancer (EORTC) 10854 randomized trial demonstrated that p53 negative tumors by IHC significantly benefit from one course of perioperative anthracycline-based chemotherapy whereas p53 positive tumors had a poor response to this therapy [24]. Furthermore, there is evidence from a recent study that tumors with normal p53 (assessed by sequencing and IHC) respond better to anthracycline and/or alkylating agents, while p53 deficient tumors respond better to taxanes [25].

bcl-2 gene encodes the Bcl-2 protein, a membrane associated protein of 26 kDa, which inhibits programmed cell death triggered by many physiological stimuli or by several stress conditions, including chemotherapy [30].

In breast cancer the prognostic and predictive value of Bcl-2 expression, alone or in correlation with p53 is still unclear [4–6, 8, 18–22, 31–34]. In general, Bcl-2 expression has been associated with favorable features (like ER positivity, low proliferative activity, differentiated tumor grade) and good prognosis [5, 8]. In several studies, Bcl-2 positivity was associated with lower risk of relapse and distant metastases or better OS [18–19], while in others was not found to be an independent prognostic factor for DFS and OS by multivariate analysis [19, 20, 31–34] or it was independent prognostic factor only in a subgroup of patients, those with positive nodes [31].

Furthermore, results on the predictive value of Bcl-2 are not yet clear [27–29, 35–36]. Two studies (one retrospective and one randomized) showed that Bcl-2 was not a predictive factor for clinical response to primary and adjuvant anthracycline based chemotherapy [27, 29]. In the adjuvant setting some retrospective studies also did not show any predictive value of Bcl-2 expression status [28], while smaller studies showed better OS in Bcl-2 positive tumors [7, 8, 35]. In a randomized study on 441 premenopausal patients with node-negative breast cancer, Bcl-2 was not found to predict response to one course of perioperative anthracycline based chemotherapy [36].

In the present study, we assessed the prognostic and predictive significance of p53 and Bcl-2 protein expression, alone and in combination, in high-risk breast cancer patients treated with dose-dense sequential chemotherapy in a randomized clinical trial (HE 10/97) investigating the role of paclitaxel (Taxol®, T) in addition to epirubicin (E) and a combination of cyclophosphamide, methotrexate and fluorouracil (CMF), [E-T-CMF versus E-CMF alone].

patients and methods

patients

Paraffin-embedded tissue blocks of primary breast cancer were prospectively collected from 397 patients that were part of the HE 10/97 trial population. This study was a prospective clinical trial coordinated and conducted by the Hellenic Cooperative Oncology Group (HeCOG). From June 1997 until November 2000, 595 eligible patients with histologically confirmed invasive breast carcinoma stage T1-3N1M0 or T3N0M0, were randomized to three cycles of epirubicin 110 mg/m^2 followed by three cycles of paclitaxel 250 mg/m^2 followed by three cycles of 'intensified' CMF (cyclophosphamide 840 mg/m^2, methotrexate 57 mg/m^2 and fluorouracil 840 mg/m^2) (E-T-CMF) or to four cycles of epirubicin, followed by four cycles of CMF (E-CMF). All cycles were given every 2 weeks. Prophylactic administration of G-CSF (Filgrastim; 5 μg/kg) and antiemetics, such as ondasetron and dexamethasone, were administered to all patients. Adaptive stratified randomization balanced by center was performed at the HeCOG Data Office, in Athens, using the following stratification factors: menopausal status (pre versus postmenopausal), hormonal receptor status (positive versus negative) and number of positive nodes (0 versus 1–3 versus ≥4). Postmenopausal were considered patients without menses for the last two years or those ≥50 years old who underwent a hysterectomy for non-malignant reasons. The clinical protocol and the accompanying translational research studies were approved by the HeCOG Protocol Review Committee and by the Institutional Review Boards of 'Kyanous Stavros' Hospital and AHEPA University Hospital. All patients provided a written informed consent for molecular studies of their tumor specimen.

Tamoxifen (TAM), 20mg daily for five years, was prescribed to 95% of the patients included in the present analysis who were estrogen and/or progesterone receptor positive or of unknown status. Ten patients (3%) did not receive adjuvant hormonotherapy due to patient refusal or disease progression. For four patients (1%) medical files were missing.

Ovarian activity was suppressed in all premenopausal patients with hormonal receptor positive tumors, by an intramuscular injection of triptoreline, 2.5 mg every month for one year. Radiation therapy was mandatory for all patients with partial mastectomy or for those with ≥4 positive lymph nodes and/or tumor size ≥ 5 cm, irrespectively of the type of initial surgical operation. TAM administration, ovarian suppression and radiation therapy were all started after the completion of adjuvant chemotherapy. After a median follow-up of 62 months there was no difference in DFS (80% versus 77%) or OS (93% versus 90%) between the two treatment groups [37]. As part of this study the results about the prognostic and predictive significance of HER-2 and VEGF expression have recently been published [38].

pathologic determinations

Primary tumor diameter and axillary nodal status were obtained from the histopathologic reports. ER and progesterone receptor (PgR) status was assessed by IHC and relative information was provided by participating institutions according to their own reference laboratories. Tissue paraffin sections stained for ER/PgR were considered as positive even when only a small number of neoplastic cells displayed nuclear immunoreaction. Histological grade was evaluated according to the Scarf, Bloom and Richardson system [39].

specimen analysis

Paraffin-embedded tissue blocks of primary breast cancers were prospectively collected and p53 and Bcl-2 expression was assessed centrally, at the Department of Pathology of 'Hygeia' Hospital, Athens, by IHC using the NeXes automated system (Ventana) in 392 and 397 patients respectively. Both p53 and Bcl-2 data were available in 375 patients. Two observers evaluated independently all IHC slides. In case of a discrepancy the two observers simultaneously reviewed the slides in order to achieve a consensus (Th.P. and A.B. for p53 and M.S. and D.V. for Bcl-2, respectively).

immunohistochemistry

Immunohistochemistry was carried out on 5 μm tissue sections from paraffin blocks using the avidin-biotin immunoperoxidase method, provided by a commercial available kit (Super Sensitive Immunodetection System, BioGenex). The following monoclonal antibodies were used: DO-1 against p53 (1:25 dilution, 1 h incubation at RT, Oncogene Science, INC, Cambridge, MA) and anti-human Bcl-2 (1:40 dilution, overnight incubation at 4°C, DAKO, Glostrup, Denmark). Briefly, the paraffin sections were deparaffinized with xylene and rehydrated through a series of descending graded ethanol. To unmask the epitopes of p53 and Bcl-2 microwave-processing pretreatment was carried out in a 10 mM citrate buffer, pH = 6.0 at 750 W for 15 min, in two cycles. Endogenous peroxidase activity was blocked by incubation for 15 min in 0.3% H_2O_2 buffer. Subsequently, biotinylated multi-link secondary antibody and avidin-biotin-complex with horseradish peroxidase were applied, followed by the addition of the chromogen (3.3′-diaminobenzidine and hydrogen peroxide). Finally, slides were counterstained with hematoxylin, dehydrated in ascending ethanol, cleared with xylene, and mounted with coverslips using a permanent mounting medium. Appropriate positive and negative controls were used in each experiment.

immunohistochemical evaluation

The slides were examined with light microscopy by two observers, who were unaware of the clinical outcome. At least 1000 cells were counted in 10 different areas in each case using the 40× objective lens. For statistical analysis purposes, the sections were scored as either negative or positive. Staining for p53 was restricted to the nucleus and the sections were graded as positive if 10% or more of the tumor cells were stained, irrespective of intensity (Figure 1). Staining for Bcl-2 was cytoplasmic and the cases were graded as positive if 5% or more of the tumor cells were stained, irrespective of intensity (Figure 2).

statistical analysis

OS was measured from the day of randomization until death due to any cause. Surviving patients were censored at the day of the last contact. DFS was measured from randomization until local recurrence, distant relapse, occurrence of contralateral breast cancer or second primary tumor or death without relapse, whichever occurred first. Time to event distributions were estimated using Kaplan-Meier curves and compared using the log-rank test. Fisher's exact test was used to investigate associations between p53, Bcl-2 and established patient and tumor characteristics.

Cox proportional hazards models were used to assess the strength of association of OS and DFS with various clinical and histologic variables in the presence of either p53 expression (negative versus positive) or in the presence of both p53 and Bcl-2 in the case of patients with estimates for both markers.

A backward selection procedure with removal criterion $p > 0.10$, identified the subclass of significant variables among the following: treatment group (E-T-CMF versus E-CMF), menopausal status (pre versus post), tumor grade (I–II versus III-undifferentiated), hormonal status (negative versus positive), tumor size (≤2 cm versus >2–5 cm versus >5 cm) and number of positive lymph nodes (0–3 versus ≥4).

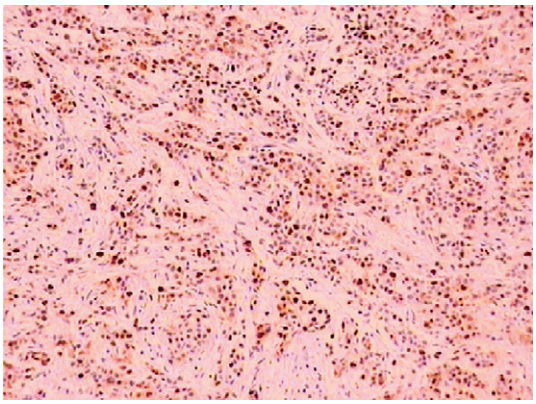

Figure 1. Intense nuclear staining for p53 in high percentage of tumor cells (×10).

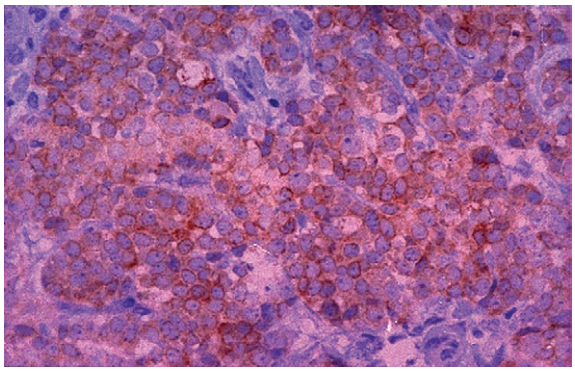

Figure 2. Strong Bcl-2 immunostaining in invasive ductal carcinoma cells (×200).

results

The present study was performed according to the guidelines of the NCI-EORTC working group on cancer diagnostics [40].

Positive expression of p53 was detected in 104 (26.5%) patients (Table 1). There were no significant differences in major characteristics, between the two treatment groups with the exception of tumor grade ($P < 0.001$) (Table 2). Positive expression of p53 was significantly associated with negative hormonal status (41% versus 19%, $P < 0.001$), worse histologic grade (70% versus 43%, $P < 0.001$), higher incidence of disease relapse (39% versus 23%, $P = 0.002$), and significantly higher rate of death (24% versus 12%, $P = 0.004$) (Table 3). The two groups of patients (positive p53 and negative p53) were balanced in terms of treatment regimen ($P = 0.73$).

After a median follow-up of 50 months (range: 0.1–75.15+), 59 (15%) patients have died. All were disease-related deaths. Overall, 107 (27%) patients have relapsed. p53 positive expression was a significant negative predictor of OS ($P = 0.002$) (Figure 3A) and DFS ($P = 0.001$) (Figure 3B). Stratifying by treatment group (E-T-CMF versus E-CMF) did not affect the results (stratified log-rank test, OS: $P = 0.001$, DFS: $P = 0.001$).

Table 1. Molecular results

	Group A E-T-CMF	Group B E-CMF
n (%)	190	202
p53 expression		
Negative	138 (73)	150 (74)
Positive	52 (27)	52 (26)
n (%)	195	202
Bcl-2 expression		
Negative	88 (45)	115 (57)
Positive	107 (55)	87 (43)
n (%)	183	192
p53 and Bcl-2 expression categories		
Positive p53 and negative Bcl-2	25 (14)	33 (17)
Positive p53 and positive Bcl-2	25 (14)	16 (8)
Negative p53 and negative Bcl-2	54 (29)	78 (41)
Negative p53 and positive Bcl-2	79 (43)	65 (34)

Results of the Cox multivariate regression analysis (Table 4) revealed that p53 positive expression (positive versus negative: HR (hazard ratio) = 2.08, 95% CI (confidence interval) 1.22–3.55, $P = 0.007$), higher number of positive nodes [≥4 versus 0–3: HR = 3.06, 95% CI 1.31–7.12, $P = 0.01$], and worse tumor grade (III–undifferentiated versus I–II: HR = 1.81, 95% CI 1.03–3.18, $P = 0.04$) were related to significantly poorer OS. The same factors were prognostic for poorer DFS, positive p53 expression (positive versus negative: HR = 1.87, 95% CI 1.25–2.79, $P = 0.002$), higher number of positive nodes (≥4 versus 0–3: HR = 3.08, 95% CI 1.69–5.63, $P < 0.001$), and worse tumor grade (III–Undifferentiated versus I–II: HR = 1.52, 95% CI 1.01–2.27, $P = 0.04$).

Negative Bcl-2 expression was detected in 203 (51%) patients (Table 1). There were no significant differences in major characteristics between the two treatment groups with the exception of grade ($P = 0.001$). Negative expression of Bcl-2 was significantly associated with negative hormonal status (31% versus 17.5%, $P = 0.002$). Of note, significantly less patients with negative Bcl-2 expression received E-T-CMF (43% versus 57%, $P = 0.02$). Bcl-2 expression did not significantly affect OS ($P = 0.10$) or DFS ($P = 0.14$) maybe due to small sample size. (Figures 4A & B).

In the population of 375 patients, with estimates for both p53 and Bcl-2 expression, the two treatment groups were balanced in terms of basic patient and tumor characteristics, except for grade ($P = 0.001$). No significant association was found between the expression of p53 and Bcl-2 ($P = 0.08$). The presence of Bcl-2 expression ($P = 0.21$) in the Cox regression models only changed the results marginally. Positive expression of p53 (HR = 1.94, $P = 0.015$), higher number of positive nodes (HR = 3.15, $P = 0.008$) and worse tumor grade (HR = 1.88, $P = 0.03$) significantly reduced patient OS. In the presence of Bcl-2 expression ($P = 0.26$), tumors with positive expression of p53 (HR = 1.83, $P = 0.004$) along with higher number of positive nodes (HR = 3.36, $P < 0.001$) and worse tumor grade (HR = 1.58, $P = 0.03$) were also associated with a significantly worse DFS. No interaction effect of p53 and Bcl-2 was found (OS: $P = 0.69$, DFS: $P = 0.27$).

Furthermore, no significant interaction of p53 expression (positive versus negative) by treatment group (E-T-CMF versus E-CMF) was found in the Cox multivariate analysis models for OS ($P = 0.89$) and DFS ($P = 0.82$). Similarly, no significant effect was found for the interaction of Bcl-2 by treatment group (E-T-CMF versus E-CMF) (OS, $P = 0.18$; DFS, $P = 0.39$). Thus, no predictive ability was established for any of the factors of interest.

Table 2. Basic patient and tumor characteristics by treatment group for patients with p53 data

	Group A E-T-CMF		Group B E-CMF	
n	190		202	
Age (years)				
Median	52		51	
Range	24–76		22–76	
	n	%	*n*	%
Menopausal status				
Premenopausal	92	48	109	54
Postmenopausal	98	52	93	46
Type of operation				
MRM	146	77	155	77
PM	44	23	47	23
Interval from operation (weeks)				
<2	11	6	17	8
2–4	97	51	87	43
>4	82	43	98	48.5
Receptor status				
Negative	49	26	48	24
Positive	140	74	153	76
Unknown	1	0.5	1	0.5
Adjuvant HT?				
No	5	4	5	3
Yes	134	95	147	96
Unknown	2	1	2	1
Tumor size				
≤2 cm	48	25	66	33
2–5	108	57	100	49
>5	34	18	36	18
Grade*				
I	10	5	4	2
II	68	36	112	55
III	110	58	86	43
Undifferentiated	2	1	–	–
Number of nodes removed				
Median	18		18	
Range	5–59		4–53	
Number of positive nodes				
Median	7		6	
Range	0–54		0–49	
0–3	41	22	61	30
≥4	149	78	141	70

*The two treatment groups are not balanced in terms of grade ($P < 0.001$).

Table 3. Basic patient and tumor characteristics by p53 expression

	p53 expression Negative (n = 288)		Positive (n = 104)	
Age (years)				
Median	52		49	
Range	22–76		24–75	
	n	%	*n*	%
Menopausal status				
Premenopausal	143	50	58	56
Postmenopausal	145	50	46	44
Treatment group				
E-T-CMF	138	48	52	50
E-CMF	150	52	52	50
Receptor status[a]				
Negative	54	19	43	41
Positive	232	81	61	59
Positive nodes				
0–3	72	25	30	29
≥4	216	75	74	71
Grade[a]				
I–II	163	57	31	30
III–undifferentiated	125	43	73	70
Tumor size				
≤2 cm	84	29	30	29
2–5	160	56	48	46
>5	44	15	26	25
Death?[b]				
No	254	88	79	76
Yes	34	12	25	24
Relapse?[c]				
No	222	77	63	61
Yes	66	23	41	39

[a]$P < 0.001$; [b]$P = 0.004$; [c]$P = 0.002$.

discussion

Adjuvant chemotherapy prolongs DFS and OS in patients with node positive early breast cancer. However, a substantial percentage of patients with clinically occult metastases at the time of diagnosis ultimately have a relapse and die in spite of receiving combination chemotherapy [1]. Prediction of response to treatment based on molecular markers was initially explored with ER levels and later with HER-2 [22, 23]. In our recently published study, no predictive ability of HER-2 status for paclitaxel treatment was evident [38]. In the present study, the expression of p53 and Bcl-2 proteins were evaluated in tumors of patients participating in a randomized trial of dose-dense sequential chemotherapy.

p53 positive expression correlated with negative hormonal status, worse histologic grade, higher incidence of disease relapse and higher death rate, as it has been previously reported by other research groups [7, 10, 12–14, 15–17, 19, 21]. p53 positive expression was a significant negative predictor of DFS ($P = 0.001$) and OS ($P = 0.002$).

Multivariate analysis showed that p53 positive expression affected negatively DFS and OS along with high nodal involvement and high histologic grade. The additional administration of paclitaxel had no influence in DFS and OS.

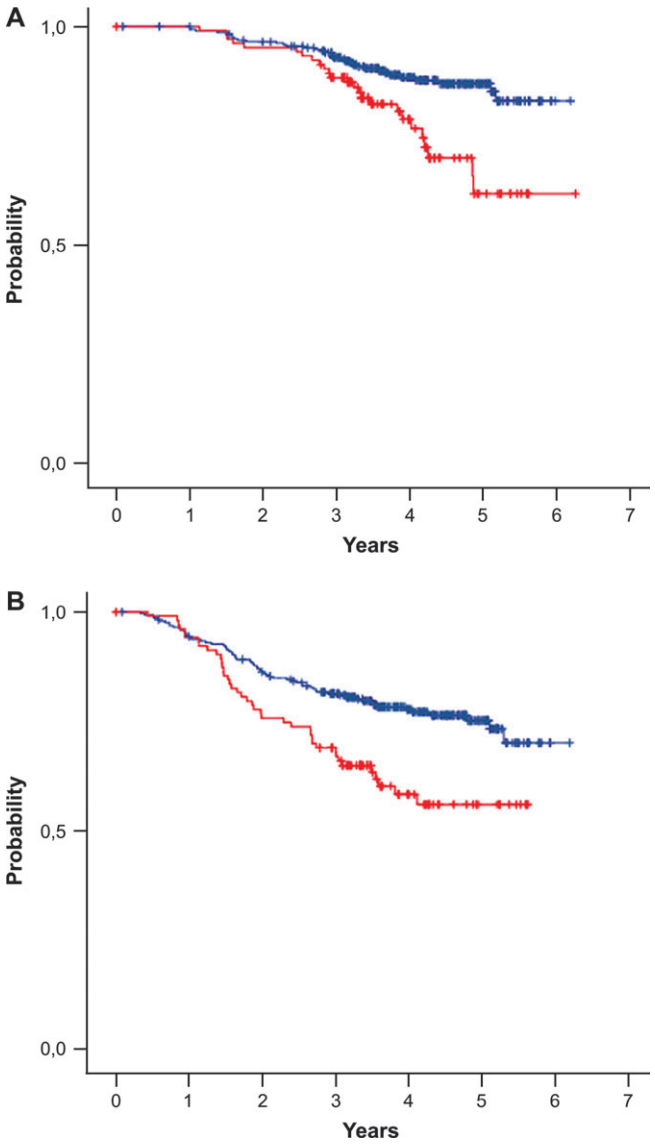

Figure 3. Overall survival (**A**) of patients with positive p53 expression (•) or with negative expression (•) ($P = 0.002$) and (**B**) Disease-free survival of patients with positive. (•) or negative (•) p53 levels ($P = 0.001$).

Furthermore, the interaction of treatment to p53 positive expression was not significant, indicating that treatment did not affect differentially DFS or OS for different p53 status. Thus, no predictive ability of p53 status for paclitaxel treatment was evident in this study. It should be noted that the cumulative dose of Epirubicin in group B was higher than in group A (440 mg/m^2 versus 330 mg/m^2). As the administration of paclitaxel in relationship of p53 has not been previously evaluated in the adjuvant setting, results from other similar studies are awaited with interest. In the neoadjuvant setting it has been suggested that the cytotoxicity of paclitaxel was related to defective p53 [25]. However, in another study in 73 patients with locally advanced breast cancer the negative expression of p53 indicates a higher chance of responding to paclitaxel followed by doxorubicin [41]. Moreover, in patients with metastatic disease the results from the correlation of the expression of p53 with response to paclitaxel were mixed

Table 4. Estimated hazard ratios (HRs) and 95% confidence intervals (CIs) for OS and DFS–multivariate analysis

	HR	95% CI	*P*-value
Overall survival			
p53 expression			
Negative	1		
Positive	2.08	1.22–3.55	0.007
Number of positive nodes			
0–3	1		
≥4	3.06	1.31–7.12	0.01
Grade			
I–II	1		
III–undifferentiated	1.81	1.03–3.18	0.04
	HR	95% CI	*p*-value
Disease-free survival			
p53 expression			
Negative	1		
Positive	1.87	1.25–2.79	0.002
Number of positive nodes			
0–3	1		
≥4	3.08	1.69–5.63	<0.001
Grade			
I–II	1		
III–undifferentiated	1.52	1.01–2.27	0.04

[42–45]. In most, p53 expression did not have any effect in treatment results [42–44].

So far, several studies reporting the correlation of p53 status with response to CMF chemotherapy, the results of which are controversial, have been published. Fewer studies reporting the benefit from an anthracycline-based chemotherapy in the adjuvant or neoadjuvant setting have also been published [22–29].

In our study negative expression of Bcl-2 was significantly associated with negative hormonal status as previously shown while it was not found to be associated with p53 expression [5, 8, 20, 29, 35].

Also, according to other reported results [19, 20, 31–34] in our study Bcl-2 expression was not determined to be a negative prognostic factor.

In the population of 375 patients, with data for both p53 and Bcl-2 expression an independent prognostic value was only found for p53 expression. Specifically, multivariate analysis revealed that higher number of positive nodes, p53 positive expression and worse tumor grade were related to significantly poorer outcome.

In a recently published analysis of the prognostic and predictive effects of p53 and Bcl-2 within a randomized trial comparing high-dose versus standard-dose chemotherapy in patients with breast cancer and ≥ 10 infiltrated lymph nodes, p53 and Bcl-2 positivity were associated with longer event-free survival [46]. Bcl-2 prognostic ability was not confirmed by our study in a larger sample, albeit treated differently.

p-53-positive patients benefited more from high-dose chemotherapy than from standard chemotherapy while p53-negative patients had more benefit from standard chemotherapy.

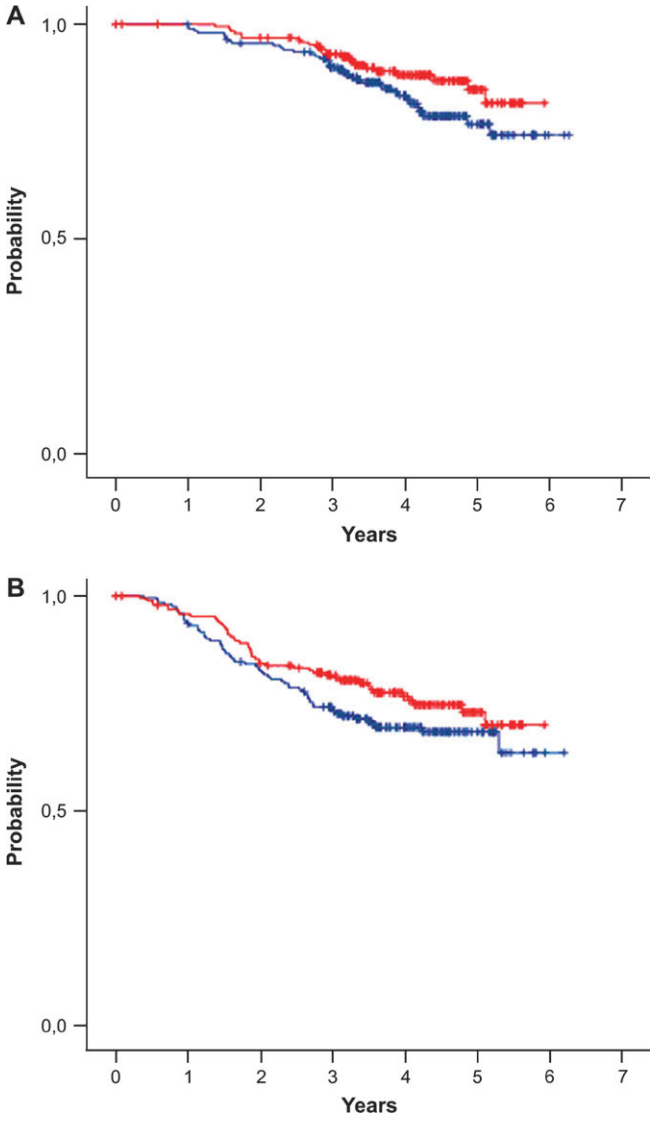

Figure 4. Overall survival (**A**) of patients with positive Bcl-2 expression (•) or with negative expression (•) ($P = 0.10$) and (**B**) Disease-free survival of patients with positive (•) or negative (•) Bcl-2 levels ($P = 0.14$).

Interestingly, Bcl-2 was found in that study to be a prognostic but not a predictive factor. On the other hand, an interaction of p53 and treatment group was evident. These results based on a relatively small number of specimens and on multiple subgroup analyses were not confirmed by our study.

Furthermore, contradictory results among different studies may be due to differences in methodology, patient populations or size of the studies, type of treatment or other unidentified causes.

In conclusion, in this group of patients treated with dose-dense sequential chemotherapy, positive expression of p53 was a significant negative prognostic factor for DFS and OS with and without adjusting for treatment group. No predictive ability of p53 or Bcl-2 status for differential response to paclitaxel treatment was evident within the 50-month follow up period our study.

appendix

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