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Human Cancer Biology

Clinical Cancer Research

ERBB2 Induces an Antiapoptotic Expression Pattern of Bcl-2 Family Members in Node-Negative Breast Cancer

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Abstract

Purpose: Members of the Bcl-2 family act as master regulators of mitochondrial homeostasis and apoptosis. We analyzed whether ERBB2 influences the prognosis of breast cancer by influencing the proapoptotic versus antiapoptotic balance of Bcl-2 family members.

Experimental Design: ERBB2-regulated Bcl-2 family members were identified by inducible expression of ERBB2 in MCF-7 breast cancer cells and by correlation analysis with ERBB2 expression in breast carcinomas. The prognostic relevance of ERBB2-regulated and all additional Bcl-2 family members was determined in 782 patients with untreated node-negative breast cancer. The biological relevance of ERBB2-induced inhibition of apoptosis was validated in a murine tumor model allowing conditional ERBB2 expression.

Results: ERBB2 caused an antiapoptotic phenotype by upregulation of *MCL-1*, *TEGT*, *BAG1*, *BNIP1*, and *BECN1* as well as downregulation of *BAX*, *BMF*, *BNIPL*, *CLU*, and *BCL2L13*. Upregulation of the antiapoptotic *MCL-1* [$P = 0.001$, hazard ratio (HR) 1.5] and *BNIP3* ($P = 0.024$; HR, 1.4) was associated with worse prognosis considering metastasis-free interval, whereas clusterin ($P = 0.008$; HR, 0.88) and the proapoptotic *BCL2L13* ($P = 0.019$; HR, 0.45) were associated with better prognosis. This indicates that ERBB2 alters the expression of Bcl-2 family members in a way that leads to adverse prognosis. Analysis of apoptosis and tumor remission in a murine tumor model confirmed that the prototypic Bcl-2 family member Bcl-x_L could partially substitute for ERBB2 to antagonize tumor remission.

Conclusions: Our results support the concept that ERBB2 influences the expression of Bcl-2 family members to induce an antiapoptotic phenotype. Antagonization of antiapoptotic Bcl-2 family members might improve breast cancer therapy, whereby MCL-1 and BNIP3 represent promising targets. *Clin Cancer Res; 16(2); 451–60. ©2010 AACR.*

ERBB2 overexpression or amplification is frequently, although not invariably, associated with poor clinical outcome (1–5). Recently, ERBB2 gene amplification has been reported to correlate with the expression of apoptosis-suppressing genes Bcl-2 and Bcl-x_L in breast cancer (6). Downregulation of Bcl-2 and Bcl-x_L with antisense oligonucleotides increased the proapoptotic activity of trastuzumab (7). This led to the hypothesis that combined targeting of ERBB2 and Bcl-2 or Bcl-x_L might represent a more effective breast cancer therapy compared with targeting only the individual factors.

Only little is known about the relationship between ERBB2 and Bcl-2 family members. Members of the Bcl-2 family act as master regulators of mitochondrial homeostasis and apoptosis (8, 9). Transfection of ERBB2 into MCF-7 breast cancer cells has been shown to upregulate the expression of Bcl-2 family members (10, 11). On the other hand, antagonization of ERBB2 by trastuzumab has

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Translational Relevance

In this article, we identified ERBB2-dependent factors of the Bcl2 family by inducibly overexpressing ERBB2 in a breast cancer cell line and by comparing these factors in ERBB2 status negative and positive breast carcinomas. The prognostic relevance of the identified ERBB2-dependent Bcl2 family members was analyzed in 782 patients with node-negative breast cancer. From a clinical standpoint, the association of high clusterin (downregulated by ERBB2) expression with lower hazard ratio for disease-free survival and time without metastasis is the most provoking result of the present study. This result is clinically relevant because phase I and II studies of agents targeting CLU are under way. The rationale is that clusterin is considered to be a cytoprotective chaperone that promotes cell survival and confers broad-spectrum treatment resistance. However, considering our data, CLU-antagonizing therapy may also eliminate a beneficial factor for spontaneous tumor development.

been reported to reduce the expression of the Bcl-2 family member MCL-1 (12). Because an antiapoptotic cross-talk between ERBB2 and antiapoptotic Bcl-2 family members, as suggested by Milella et al. (7), is of high clinical relevance, we addressed this topic using three different approaches. First, we used a MCF-7 breast cancer cell line conditionally expressing ERBB2 (13) and observed that ERBB2 upregulated antiapoptotic and downregulated proapoptotic Bcl-2 family members. Second, we analyzed the clinical relevance of ERBB2-dependent Bcl-2 family members in a cohort of 782 patients with breast cancer and observed an association with poor prognosis. Third, we validated the clinical observations in a mouse tumor model (14) showing that antiapoptotic Bcl-x_L partially substitutes for ERBB2 in survival signaling of experimental tumors *in vivo*. Taken together, these findings show the clinical relevance of ERBB2-dependent modulation of apoptosis-regulating Bcl-2 family proteins.

Materials and Methods

Cells with ERBB2 expression under the control of tTA. NIH3T3, an immortalized cell line originally derived from mouse embryo fibroblasts, was obtained from American Type Culture Collection. Wild-type NIH3T3 and its derivatives were grown in DMEM (PAN) supplemented with 10% fetal bovine serum (tetracycline free; Clontech), and 1% penicillin-streptomycin (PAN). Cells were cultured at 37°C in 5% CO_2 humidified air. Conditional expression of ERBB2 was achieved by using the Tet-off system originally described by Gossen et al. (15, 16). Briefly, wild-type NIH3T3 cells were cotransfected with three vectors (pUHD 15-1, pTBC1 Hygro, and pTBC HER2/SEAP) as described by Baasner et al. (17), resulting in a cell line termed NIH3T3-HER2.

Cells with conditional ERBB2 expression and constitutive $Bclx_L$ expression. The pM_bcl-xl_pie (MS87) vector (Supplementary Fig. S1) was transfected into NIH3T3-HER2 cells resulting in a cell line named NIH3T3-HER2-$Bclx_L$ constitutively expressing $Bclx_L$. The antiapoptotic cDNA $Bclx_L$ was subcloned into the vector pMxIG (18), generating the pM_Bcl-xl_pie vector and the insert was confirmed by sequencing (19). The same culture conditions were used as for the NIH3T3-HER2 cells. The replication-defective recombinant retrovirus vector pMxIG expresses green fluorescent protein (18) to be able to monitor transduction efficacies by parallel experiments with flow cytometric detection of green fluorescent protein expression (20). Besides the pMxIG vector containing the $Bclx_L$ construct, NIH3T3-HER2 cells were cotransfected with the vector without cDNA resulting in a control cell line named NIH3T3-HER2-MOCK. Transfection and culture conditions were the same as for the NIH3T3-HER2-$Bclx_L$ cells.

Mouse tumor model. NIH3T3-HER2 cells (7×10^6) or derivatives of them were s.c. injected into the dorsal skin of 3- to 4-wk-old male nude mice (cd *nu*$^-$/*nu*$^-$; Charles River). The experimental design is shown in Fig. 1. A previously described mouse tumor model was applied in which ERBB2 expression is controlled by the Tet-off system (14). The expression of ERBB2 in these mouse tumors was downregulated by s.c. injection of 10 mg of anhydrotetracycline/kg body weight into the nude mice. For Western blot analysis, tumors were treated for 1, 3, and 7 d with anhydrotetracycline. Thereafter, mice were sacrificed by cervical dislocation. Tumors were isolated as described previously (14).

Western blot analysis. Western blot analysis was done as previously detailed (2). Primary antibodies: Bcl-x_L, caspase-3, and ERBB2 (Cell Signaling Technology, New England Biolabs GmbH) were used at a dilution of 1:1,000 and β-actin (Sigma) at a dilution of 1:2,000. Secondary antibodies: rabbit anti-mouse IgG (Sigma) and goat anti-rabbit IgG (Cell Signaling Technology) were used at a dilution of 1:5,000. Primary and secondary antibodies were diluted in PBST containing 10% Roti-Block.

Cultivation of MCF-7-NeuT cells and analysis of RNA expression patterns. The MCF-7 breast carcinoma cell line was obtained from American Type Culture Collection, cultured at 37°C in a humidified 5% CO_2 air atmosphere, and transfected with pINSpBI-NeuT/EGFP and pcDNA3Neo/rtTA2 as described by Trost et al. (13). NeuT expression, an oncogenic version of ERBB2, was induced by doxycycline (obtained as the hydrochloride salt from Sigma) at a final concentration of 1 μg/mL in all experiments. The generated MCF-7-NeuT cells were exposed to doxycycline for periods of 0, 6, 12, and 24 h as well as 3 and 14 d. Three independent repeat experiments were done. Between each repeat experiment, the MCF-7-NeuT cells were cultivated for at least two passages. Cells were harvested using TRItidy G-Reagent (AppliChem GmbH) and subsequently RNA was isolated according to the protocols of the

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manufacturer and stored at −80°C. Before microarray analysis, RNA integrity and concentration were examined using an Agilent 2100 Bioanalyzer (Agilent Technologies). We used the RNA 6000 LabChip Kit (Agilent Technologies) according to the instructions of the manufacturer. Microarray analysis was conducted at the microarray core facility of the Interdisziplinäres Zentrum für klinische Forschung, Leipzig, Germany (Faculty of Medicine, University of Leipzig). Immunoblot analysis was done as described previously (13, 21).

Patient characteristics, tissue specimens, and gene array analysis. Three recently described cohorts of 782 patients with node-negative breast cancer, who did not receive chemotherapy, were analyzed (1). Patient characteristics, including the frequency of ERBB2, estrogen receptor (ER), as well as progesterone receptor (PR) have already been published by our group (1). The combined cohort consisted of three subcohorts, the Mainz (n = 194), the Rotterdam (n = 286) and the Transbig (n = 302) cohorts. The Mainz study cohort (1), the only cohort for which multivariate analysis were done in this study, consisted of 200 consecutive lymph node-negative breast cancer patients treated at the Department of Obstetrics and Gynecology of the Johannes Gutenberg University Mainz between 1988 and 1998 (1). Six borderline cases were not included in the present study resulting in a case number of 194. Patients in the Mainz cohort were all treated with surgery and did not receive any systemic therapy in the adjuvant setting. The established prognostic factors (histologic grade, tumor size, and age at diagnosis) were collected from the original pathology reports of the gynecologic pathology division within our department. Patients' characteristics have been published by Schmidt et al. (1). For all tumors, samples were snap-frozen and stored at −80°C. Gene expression profiling of the patients' RNA was done using the Affymetrix HG-U133A array and the GeneChip System as described (1). Results obtained from the Mainz cohort were validated in two previously published microarray data sets. Two breast cancer Affymetrix HG-U133A microarray data sets including patient outcome information were downloaded from the National Center for Biotechnology Information GEO data repository. The first data set, the Rotterdam cohort (22), represents 180 lymph node–negative relapse-free patients (GSE2034) and 106 lymph node–negative patients that developed a distant metastasis. None of these patients had received systemic neoadjuvant or adjuvant therapy (Rotterdam cohort). The original data were recalculated to a TGT of 500. The second data set, the Transbig cohort, consists of 302 samples from patients with breast cancer that remained untreated in the adjuvant setting after surgery (23,24). GSM numbers of samples (from GSE6532 and GSE7390) used for analysis are listed in the Supplementary Tables previously published by Schmidt et al. (1). Raw.cel file data were processed by MAS 5.0 using a target value (TGT) of 500.

ERRB2 and ER as well as PR status of the Mainz cohort were defined by immunostaining and in case of ERBB2 by combined immunostaining and fluorescence *in situ* hybridization as described below. Immunohistochemical analyses were done on 4-μm-thick sections according to standard procedures. Serial sections of formalin-fixed and paraffin-embedded tumor tissues were stained with monoclonal ER antibodies (clone 1D5, Dako), monoclonal PR antibodies (clone PgR 636, Dako), as well as polyclonal ERBB2 antibodies (A0485, Dako). The sections were incubated with a biotin-labeled secondary antibody and streptavidin-peroxidase for 20 min each, then for 5 min with 0.05% 3′3-diaminobenzidine-tetrahydrochloride, and finally counterstained with hematoxylin. All series included appropriate positive and negative controls and all controls gave adequate results. ERBB2 was scored from 0 to 3+ according to the instructions published by the manufacturer. All ERBB2 2+ cases were confirmed by fluorescence *in situ* hybridization using a dual-color probe

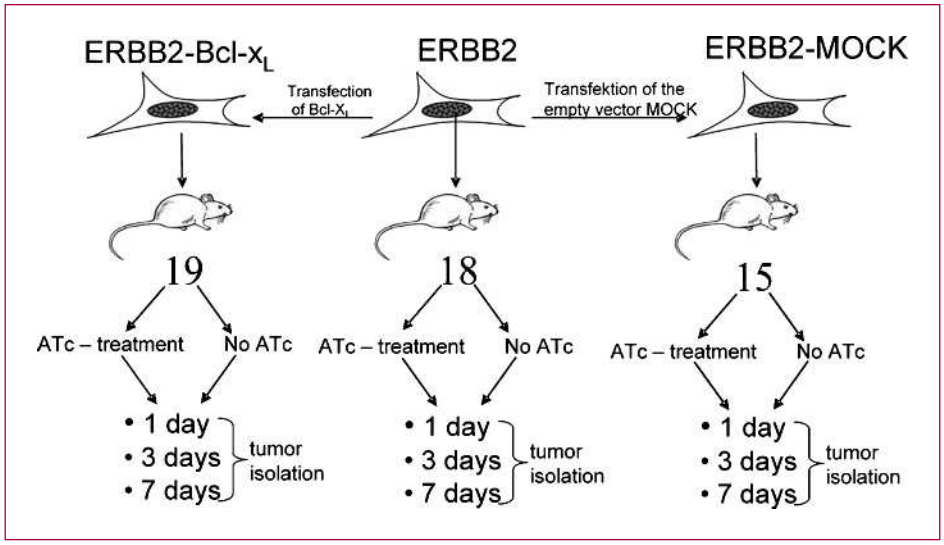

Fig. 1. Experimental design of the mouse tumor experiments. Nude mice received s.c. injections of NIH3T3 cells expressing ERBB2 (*ERBB2*), ERBB2 and Bcl-x_L (*ERBB2-Bcl-x_L*), as well as ERBB2 expressing cells mock transfected with an empty vector (*ERBB2-MOCK*). As soon as the individual tumors reached a volume of 1.3 cm^3, ERBB2 was downregulated by the Tet-off system. Subsequently, the tumor volume was monitored (Fig. 3) and tumor tissue was analyzed by immunoblot (Fig. 2).

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Table 1. Induced expression of NeuT, an oncogenic version of ERBB-2, causes predominantly anti-apoptotic alterations in RNA expression of Bcl-2 family members and proteins interacting with Bcl-2 family members

Symbol (gene)	Function	Expression after induction of NeuT* (fold expression)				
		6 h	12 h	24 h	3 d	14 d
Bcl-2 family members						
MCL-1 (myeloid cell leukemia 1)	Antiapoptotic protein of the Bcl-2 family that heterodimerizes with other Bcl-2 family members to protect against apoptotic cell death (37)	2.519	3.106	2.198	2.319	1.864
BMF (BCL2-modifying factor)	Interaction with prosurvival Bcl-2 family members to trigger apoptosis (38)	−1.576	−1.590	−1.577	−1.315	−1.205
BCL2L13 (BCL2-like 13)	Inhibits cell death upon growth factor withdrawal similar to Bcl-2 (39)	−1.355	−1.321	−1.279	−1.133	−1.056
BNIP1 (BCL2/adenovirus E1B 19-kDa protein-interacting protein 1)	BNIP1 variants interact with Bcl-2 and also with Bcl-xL (30, 33)	1.973	1.844	1.559	1.787	1.052
BNIP3 (BCL2/adenovirus E1B 19-kDa protein-interacting protein 3)	Bcl-2 proapoptotic family member recently shown to induce necrosis rather than apoptosis. Interacts with other Bcl-2 family members (31–33)	1.023	1.202	1.669	1.017	1.043
BAX (BCL2-associated X protein)	Induces apoptosis by binding to the mitochondrial permeability transition pore complex (37, 40)	1.056	−1.237	−1.204	−1.424	−1.586
Direct protein interaction with Bcl-2 family members						
BNIPL (BCL2/adenovirus E1B 19-kDa protein-interacting protein 2-like)	BCH domain of the BNIPL2 i soform interacts with Bcl-2, proapoptotic function (41)	−1.383	−2.062	−1.814	−1.685	−2.202
TEGT (testis-enhanced gene transcript)	Interactions between the proteins of the TEGT and the Bcl-2 families, stimulation of the antiapoptotic function of Bcl-2 or inhibition of the proapoptotic effect of BAX (42)	0.012	0.068	−0.012	1.571	1.545
CLU (clusterin)	Inhibits apoptosis by interfering with BAX (26)	1.050	1.257	−1.013	−4.095	−4.323
BECN1 (beclin 1)	Beclin interacts with Bcl-2 and BCLXL but not with BAX (22)	1.438	1.552	1.367	1.419	1.308
BAG1 (BCL2-associated athanogene 1)	Binds to Bcl-2, prevents fibroblasts from undergoing apoptosis (43, 44)	1.654	2.020	1.558	1.089	1.088

NOTE: NeuT was expressed in MCF-7 breast cancer cells in a doxycycline-dependent manner using the Tet-on system. RNA from MCF-7 cells was harvested 6, 12, and 24 h as well as 3 and 14 d after onset of exposure to doxycycline and analyzed by Affymetrix gene array. Three independent batches of MCF-7 cells were analyzed, in which the cells were cultured for at least two passages between the individual experiments and doxycycline incubations, as well as RNA isolations which were done at different days. Expression data are medians of the corresponding probe sets. We summarized all Bcl-2 family members and all factors directly interacting with Bcl family members that were upregulated or downregulated by a factor of at least 1.5 as a consequence of induced NeuT overexpression.

*Expression of MCF-7 cells not exposed to doxycycline was used as a control and corresponds to a gene expression of 1.0.

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(DakoCytomation) containing a spectrum orange–labeled ERBB2 gene (17q11.2-q12) probe and a spectrum green–labeled centromere control for chromosome 17 (17p11.1-q11.1). Appropriate positive controls were included into each staining run. Analysis was done with a Zeiss fluorescence microscope (Axioskop 2). A minimum of 80 non-overlapping nuclei were evaluated and the ratio of ERBB2 signals per nuclei relative to chromosome 17 centromere signals was calculated. Ratios of 2 and more were classified as ERBB2 amplification. ERBB2 2+ tumors with ERBB2 amplification were finally considered HER-2 positive. ER and PR were assessed using an immunoreactive score defined by the product of a proportion score (0, none; 1, <10%; 2, 10-50%; 3, 51-80%; 4, >80% positive cells) and an intensity score (0, no staining; 1, weak; 2, moderate; 3, strong). Only nuclear staining was considered for ER and PR. Patients with a score of >1 were considered positive for ER or PR, respectively.

Survival analysis. Univariate and multivariate Cox models were applied to analyze a possible association between RNA expression of Bcl-2 family members and prognosis. RNA expression levels of Bcl-2 family members were evaluated as continuous variables (log 2 transformed data). Disease-free survival (DFS) was computed from the date of diagnosis to the date of local recurrence of disease, distant metastasis, cancer of the contralateral breast, or death from cancer. The metastasis-free survival interval (MFI) was computed from the date of diagnosis to the date of diagnosis of distant metastasis. Patients who died of an unrelated cause were censored at the date of death. Survival times were compared using Kaplan-Meier plots and the log rank test. Dichotomization was done as follows: ERBB2 status in positive and negative, hormone receptor status in positive (ER and/or PR positive) and negative (both, ER and PR negative), histologic grade in GIII versus GI and GII, pT stage in pT_2 and pT_3 (> 2 cm) versus pT_1 (≤ 2 cm). Correlations were analyzed using the Spearman correlation test. All *P* values were two-sided. As no correction for multiple testing was done, they should be regarded as descriptive measures. All analyses were done using SPSS17.0.

Results

ERBB2 expression induces an antiapoptotic phenotype in breast cancer cells. To study the influence of ERBB2 expression on Bcl-2 family members, we used an MCF-7 cell line (MCF-7-NeuT) that allows doxycycline-induced expression of NeuT, an oncogenic version of ERBB2 (13, 25). MCF-7-NeuT cells were exposed to doxycycline for 6, 12 and 24 hours as well as 3 and 14 days. RNA was harvested from three independent biological replicates and was used for Affymetrix gene array analysis. The number of genes altered by at least a factor of 1.5 after 6, 12, and 24 hours, 3 and 14 days following induction of NeuT amounted to 30, 103, 143, 208, and 223. Among these genes were five members of the Bcl-2 family, i.e., *MCL-1*, *BMF*, *BCL2L1*, *BNIP3*, and *BAX*, as well as five direct protein interaction partners with Bcl-2 family members (Table 1). The strongest induction was observed for the antiapoptotic *MCL-1*, whereas the proapoptotic factors *BAX* and *BMF* were markedly downregulated. Further upregulated factors with antiapoptotic functions were *TEGT*, *BAG1*, *BNIP1*, and *BECN1*, although a role of the latter in apoptosis is still discussed controversially (22). Further downregulated factors with

Table 2. Univariate Cox analysis of the prognostic influence of BNIP3, CLU, and BCL2L13 in three cohorts of untreated node-negative breast cancer

Bcl-2 family–associated factor		Mainz* cohort (*n* = 194)	Rotterdam cohort (*n* = 286)†	Transbig cohort (*n* = 302)†	Combined cohorts (*n* = 782)†
BNIP3	*P* value	*0.048*	*0.024*	0.814	*0.082*
	HR	*1.409*	*1.394*	1.034	*1.164*
	95% CI	*1.004-1.978*	*1.044-1.861*	0.781-1.370	*0.981-1.381*
CLU	*P* value	*0.001*	*0.006*	0.721	*0.008*
	HR	*0.705*	*0.813*	1.029	*0.884*
	95% CI	*0.569-0.873*	*0.703-0.941*	0.880-1.202	*0.806-0.969*
BCL2L13	*P* value	*0.019*	*0.049*	0.161	0.265
	HR	*0.453*	*0.651*	1.574	0.830
	95% CI	*0.235-0.876*	*0.424-0.998*	0.835-2.964	0.598-1.152

NOTE: The three studied Bcl-2 family members amounted to statistical significance in two of three cohorts. Results with $P < 0.1$ values are italicized.

Abbreviation: 95% CI, 95% confidence interval.

*DFS was analyzed in the Mainz cohort. The respective data of MFI are given in Supplementary Table 6.

†MFI is given for the Rotterdam and Transbig cohorts.

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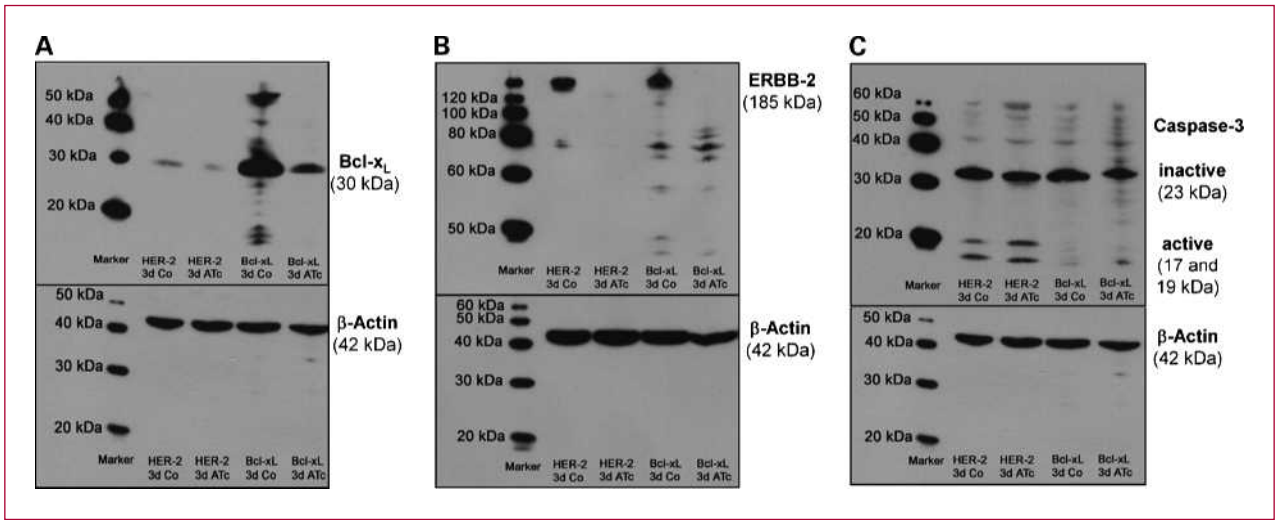

Fig. 2. Expression of Bcl-x_L (*A*), ERBB2 (*B*), and active as well as inactive caspase-3 (*C*) in tumor tissue of nude mice after injection of the NIH3T3-ERBB2-Bcl-x_L cells (*Bcl-x_L*) or NIH3T3-ERBB2 (parental) cells (ERBB2). Tumor tissue was analyzed 3 d after onset of daily anhydrotetracycline injections (*3d ATc*) and was compared with time-matched control tumors (*3d Co*). Tumors resulting from injection of NIH3T3-ERBB2-Bcl-x_L cells expressed high levels of Bcl-x_L in contrast to the parental NIH3T3-ERBB2 cells (*A*). The extent of ERBB2 downregulation by anhydrotetracycline (*ATc*) was similar in both tumor types (*B*). Importantly, lower levels of active caspase-3 were observed in tumors expressing Bcl-x_L (*C*). This was the case after anhydrotetracycline administration, but also in mice that did not receive anhydrotetracycline, showing that tumors from NIH3T3-ERBB2 cells have relatively high levels of spontaneous apoptosis.

possible proapoptotic functions are *BNIPL* and *CLU* (Table 1). With the exception of *BNIP3*, whose function as a bona fide proapoptotic BH3 protein still remains to be genetically proven, all other genes fit into a pattern of upregulation of antiapoptotic and downregulation of proapoptotic factors.

Correlation of ERBB2-associated Bcl-2 family members with clinical outcome in node-negative breast cancer. It should be considered that ERBB2-induced alterations in the expression of Bcl-2 family members were obtained in the breast cancer cell line MCF-7, which may not be representative for a population of breast cancer patients. Therefore, we studied a possible correlation between ERBB2 RNA expression and all Bcl-2 family members in the tumor tissue of 782 patients with breast cancer. Interestingly, four of five Bcl-2 family members that were upregulated in MCF-7 cells after the induction of ERBB2, i.e., *TEGT*, *BAG1*, *BNIP1*, and *BECN1* (Table 1), were also positively correlated with ERBB2 in at least one of the three cohorts of patients with breast cancer (Supplementary Table S1). In addition, *BAX* was negatively correlated with ERBB2 in the Transbig cohort ($P = 0.003$), confirming the negative association between ERBB2 and *BAX* observed in MCF-7 cells.

Because 10 Bcl-2 family–related RNA species showed altered expression levels after NeuT induction in MCF-7 cells, we next set out to analyze the potential clinical relevance of these factors. For this purpose, we used three cohorts of medically untreated patients with node-negative breast cancer ($n = 782$) that have been analyzed by gene expression arrays: the Mainz cohort (1), the Rotterdam cohort (22), and the Transbig cohort (23, 24). Univariate analysis showed that high expression of BNIP3 was associated with worse prognosis in the Mainz and Rotterdam cohorts (Table 2). High expression of proapoptotic clusterin (*CLU*) was associated with better prognosis in the Mainz and Rotterdam cohorts and in the combined cohort of 782 patients (Table 2). MCL-1 and *BNIP1* were associated with worse prognosis in the combined cohort of 782 patients, but not in the individual cohorts (Supplementary Table S2). Multivariate analysis of the Mainz cohort revealed *CLU* as a prognostic factor independent from the established clinical variables age, pT stage, histologic grade, ER and PR as well as ERBB2 status [$P = 0.020$; hazard ratio (HR), 0.780; 95% confidence interval, 0.633-0.961; Supplementary Table S3A]. *BNIP3* expression was associated with histologic grading (Supplementary Fig. S2) and, therefore, no independent prognostic factor (Supplementary Table S3B). In summary, ERBB2 expression correlates with a pattern of Bcl-2 family members that is associated with worse prognosis. This includes downregulated *CLU*, upregulated *BNIP3*, and upregulated *MCL-1* and *BNIP1* which are associated with worse prognosis in ERBB2-positive and ERBB2-negative breast cancers.

Role of additional Bcl-2 family members in breast cancer and their relation to ERBB2. In the previous paragraphs, we have focused on Bcl-2 family members that were regulated by ERBB2 in MCF-7 cells. Next, we systematically studied the prognostic relevance of all Bcl-2 family members (listed in Supplementary Table S4). However, apart from the Bcl-2 family members already described in the previous paragraphs, only one additional relevant factor, i.e., *BCL2L13* could be identified (Table 2). High expression of *BCL2L13* was associated with a decreased HR for

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MFI in the Mainz and in the Rotterdam cohorts. Due to the lack of published clinical data in the Rotterdam cohort, BCL2L13 could be tested in the multivariate Cox model only for the Mainz patients. Here, it was a prognostic factor independent from the established clinical factors age, grading, pT stage, ERBB2, and hormone receptor status (Supplementary Tables S3C and S5 for DFS and MFI).

A negative correlation between ERBB2 and BCL2L13 expression ($P = 0.017$) was found in the Mainz cohort (Supplementary Table S1). Therefore, BCL2L13 represents another Bcl-2 family member, which is possibly modified by ERBB2 in a way that may be associated with worse prognosis.

Bcl-2 family member Bcl-x_L partially substitutes for ERBB2 and antagonizes the success of proapoptotic ERBB2 blocking therapy. Our expression analysis suggests that the modulation of Bcl-2 family proteins is important for the biological function of the ERBB2 oncogene. To address this hypothesis at a functional level, we used a tumor model in which tetracycline-controlled downregulation of human ERBB2 causes apoptosis in tumor cells leading to a rapid tumor remission (2, 14, 17, 24). To dissect the role of apoptosis-regulating factor in ERBB2 signaling *in vivo*, antiapoptotic Bcl-x_L was expressed in conditional NIH3T3-ERBB2 cells. Bcl-x_L was chosen for this experiment because it represents one of the best-understood Bcl-2 family members acting similarly as MCL-1 and Bcl-2. After injection of the (*a*) NIH3T3-ERBB2-Bcl-x_L cells, (*b*) NIH3T3-ERBB2 (parental) cells, (*c*) NIH3T3-ERBB2-MOCK (expressing a control vector) into nude mice, all three cell lines formed rapidly proliferating tumors within 14 days (Experimental Design: Fig. 1). Tumors resulting from the injection of NIH3T3-ERBB2-Bcl-x_L cells expressed high levels of Bcl-x_L in contrast with the parental NIH3T3-ERBB2 and NIH3T3-MOCK cells (Fig. 2A; Supplementary Fig. S3A). Downregulation of ERBB2 by anhydrotetracycline was equally effective in all three cell types (Fig. 2B; Supplementary Fig. S3B), and did not influence the levels of Bcl-x_L (Fig. 2A, Supplementary Fig. S3A).

Next, we studied whether Bcl-x_L affects the tumor response upon withdrawal of ERBB2 *in vivo* using the s.c. injection of NIH3T3-ERBB2-Bcl-x_L, NIH3T3-ERBB2, or NIH3T3-ERBB2-MOCK cells into nude mice. As soon

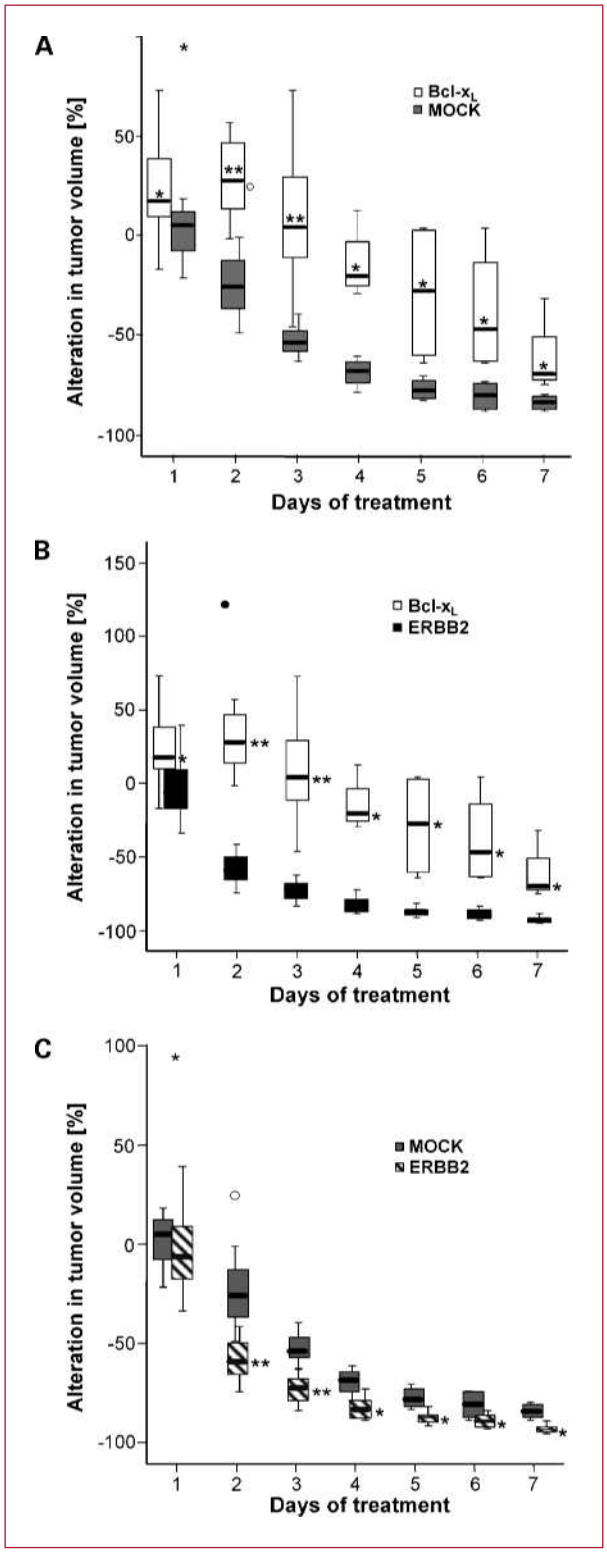

Fig. 3. Bcl-x_L expression in tumors antagonizes the therapeutic efficiency of ERBB2 downregulation. Subcutaneous tumors were induced in nude mice by injection of NIH3T3 cells expressing ERBB2, ERBB2 and Bcl-x_L, as well as cells expressing ERBB2 mock-transfected with an empty vector. Downregulation of ERBB2 was achieved by daily injections of anhydrotetracycline for 7 d. The alteration of tumor volume compared with the day of the first anhydrotetracycline injection (day 0) is given. *A*, NIH3T3 cells expressing ERBB2 and Bcl-x_L (*Bcl-x_L*) versus NIH3T3 cells expressing ERBB2 and mock-transfected with an empty vector (*MOCK*). *B*, NIH3T3 cells expressing ERBB2 and Bcl-x_L (*Bcl-x_L*) versus NIH3T3 cells expressing ERBB2 (*ERBB2*). *C*, NIH3T3 cells expressing ERBB2 and mock-transfected with an empty vector (*MOCK*) versus NIH3T3 cells expressing ERBB2 (*ERBB2*). Comparing mock-transfected cells to the parental NIH3T3-ERBB2 cells showed that the retroviral transduction itself might have induced a slight increase in resistance (*C*). However, this small difference cannot explain the much larger effect observed in the presence of Bcl-x_L (*A* and *B*).

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as the individual tumors reached a volume of 1.3 cm^3, mice received injections of anhydrotetracycline for 7 subsequent days to downregulate ERBB2 transgenic expression. As observed in previous studies (2, 14, 17, 21), withdrawal of ERBB2 caused strong tumor remissions. Interestingly, remissions of tumors from NIH3T3-ERBB2-Bcl-x_L cells were less pronounced as compared with the NIH3T3-ERBB2-MOCK control tumors (Fig. 3A) and parental NIH3T3-ERBB2 (Fig. 3B) tumors. Previously, we have shown that apoptosis contributes to tumor remission after withdrawal of ERBB2 (14, 21). Therefore, we studied cleaved (active) caspase-3 in tumors after 3 days of anhydrotetracycline administration (Fig. 2C). Higher levels of cleaved caspase-3 were observed in tumors from NIH3T3-ERBB2 cells as compared with NIH3T3-ERBB2-Bclx$_L$ cells. In conclusion, Bcl-x_L counteracted tumor remissions of an ERBB2-antagonizing treatment by antagonizing apoptosis in tumor cells.

Discussion

To better understand the relationship between ERBB2 and Bcl-2 family members, we first studied the extent to which the expression of Bcl-2 family members is influenced by conditional ERBB2 expression in the human breast cancer cell line MCF-7, and subsequently, analyzed the prognostic relevance of the identified genes in patients with breast cancer. Inducible expression of NeuT, an oncogenic version of ERBB2, was achieved by the Tet-on system (13, 25), and ERBB2 expression levels were comparable with breast tumors scoring 2+/3+. The induced ERBB2 was functional as evidenced by increased phosphorylation of ERK1/2, Akt, and P38 after induction of the Tet-on system by doxycycline. Time-dependent gene array analysis was done ranging from 6 hours to 14 days after switching on NeuT expression. NeuT expression induced an antiapoptotic expression pattern of Bcl-2 family members in which the antiapoptotic *MCL-1*, *TEGT*, *BAG1*, *BNIP1*, and *BECN1* were reproducibly upregulated and four proapoptotic Bcl-2 family members and related genes were downregulated, i.e., *BAX*, *BMF*, *BNIPL*, and *CLU* (Table 1). Although the role of CLU in apoptosis is still discussed controversially, overexpression has been reported to induce cell death in tumor cells (26). In conclusion, genome-wide analysis of RNA expression following the induction of oncogenic ERBB2 transcription results in the expression of an antiapoptotic phenotype.

It should be considered that the association between ERBB2 and Bcl-2 family members was observed in a single breast cancer cell line, which may not be representative for cohorts of patients. However, when we studied three independent cohorts of node-negative breast cancer patients (n = 782), all five upregulated antiapoptotic factors (MCL-1, *TEGT*, *BAG1*, *BNIP1*, and *BECN1*) observed in NeuT expressing MCF-7 cells showed a correlation with ERBB2 in at least one of the cohorts. Also, the negative correlation between ERBB2 and *BAX* was confirmed.

Having identified 10 ERBB2-associated Bcl-2 family members, we next examined their prognostic relevance in patients with breast cancer. For this purpose, we used Affymetrix gene array data from 782 patients with node-negative breast cancer. Because established clinical prognostic variables such as age, pT stage, grading, hormone receptor status, and ERBB2 status were only available in the Mainz cohort, multivariate Cox analysis was only done in the latter. High expression of proapoptotic clusterin (*CLU*), which has been shown to be negatively correlated with ERBB2 in MCF-7 cells, was associated with better prognosis in the combined cohort, and in the Mainz as well as the Rotterdam cohorts. Multivariate analysis showed that *CLU* expression is a prognostic factor independent from established clinical variables (Supplementary Table S3A). Because *CLU* was downregulated in MCF-7 breast cancer cells in response to ERBB2 induction, this is one of several ERBB2-dependent Bcl-2 family members that correlate with adverse prognosis. From a clinical standpoint, the association of high clusterin expression with longer DFS and time without metastasis is the most provoking result of the present study. The association of *CLU* with better prognosis was observed in the Mainz cohort (P = 0.001), the Rotterdam cohort (P = 0.006), and in the combined cohort of 782 patients (P = 0.008). Thus, from a statistical point of view, the positive prognostic influence of *CLU* is very convincing. This result is clinically relevant because phase I and II studies of agents targeting CLU are under way (27–29). The rationale is that clusterin is considered to be a cytoprotective chaperone that promotes cell survival and confers broad-spectrum treatment resistance (27, 29). Indeed, several studies have shown that silencing clusterin may enhance the cytotoxicity of several chemotherapeutic agents (for a review, see ref. 29). However, considering the presence of two *CLU* isoforms, a nuclear form, that is proapoptotic, and a secretory form, that is prosurvival, and bearing in mind that some cancers show decreased levels of secretory *CLU* (29), the role of clusterin in tumor development is not yet understood. Because our study includes a higher number of cases (n = 782) as compared with previous studies (n = 141 and n = 158; refs. 30, 31, respectively), the reported trends towards worse prognosis for patients with high *CLU* expression should be carefully re-evaluated. It should be considered that *CLU*-antagonizing therapy might, on the one hand, improve the sensitivity of tumor cells to cancer chemotherapy, but might also eliminate a beneficial factor for spontaneous tumor development. In our study, none of the patients had received chemotherapy, thus focusing on the role of *CLU* on the natural course of the disease.

BNIP3 was the only factor which, at first glance, did not fit the scenario that ERBB2 induces an antiapoptotic phenotype because *BNIP3* was upregulated in response to ERBB2 induction in MCF-7 cells and it is considered to represent a proapoptotic Bcl-2 family member (32–34). However, high BNIP3 expression was associated with worse prognosis in the Mainz and Rotterdam cohorts. Therefore, if the role of BNIP3 in breast cancer is really proapoptotic, its negative

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prognostic influence would be counterintuitive. Moreover, genetic and clear biochemical confirmation of BNIP3's putative role as a BH3-only protein is still awaited. Recently, conflicting results have been reported for immunohistochemically determined BNIP3, which has been reported to be associated with good survival in invasive carcinoma but with increased risk of recurrence in ductal carcinoma *in situ* (35). Our results clearly show that high expression of *BNIP3* is associated with worse prognosis and may therefore be evaluated as a possible therapeutic target.

MCL-1, which was upregulated in MCF-7 cells following the expression of NeuT, was associated with adverse prognosis in the combined cohort, but not in the individual cohorts. Therefore, confirmation in further studies is required. All other ERBB2-associated Bcl-2 family members were not consistently associated with prognosis.

It should be considered that the 10 ERBB2-associated genes in Table 1 were identified in a single cell line (MCF-7). Although the ERBB2 dependence of the 10 genes has formally been proven in this particular cell line, it is unlikely that the result is representative for populations of patients. Therefore, we systematically studied the relevance of all known Bcl-2 family members and related genes.[7] Analyzing all 28 Bcl-2 family members or associated factors that were present on our chip (Supplementary Table S4), we identified only one additional factor consistently associated with prognosis: *BCL2L13*, which was also negatively correlated with ERBB2 expression in breast cancer tissue. High expression of *BCL2L13* was associated with better prognosis. Interestingly, the prognostic value of *BCL2L13* was independent from the established clinical factors in the multivariate Cox analysis. BCL2L13 (Bcl-rambo) has not yet been studied in breast cancer. To our knowledge, the only publication reporting a correlation of BCL2L13 with prognosis in neoplastic diseases is a study in acute lymphoblastic leukemia, in which BCL2L13 was associated with L-asparaginase resistance (36). BCL2L13 is a proapoptotic member of the Bcl-2 family (37, 38). Therefore, it seems to fit our general pattern that high *BCL2L13* expression was associated with better prognosis.

To functionally validate a role for antiapoptotic Bcl-2 family members in oncogenic ERBB2 signaling *in vivo*, we used a murine tumor model that allows the study of ERBB2-dependent tumors (2, 14, 17, 21). Switching off ERBB2 expression in these tumors leads to apoptosis and tumor remission *in vivo*. When we additionally overexpressed Bcl-x_L in the tumor cells, apoptosis and tumor remission were clearly reduced in response to the withdrawal of ERBB2. This shows that the Bcl-2 family member Bcl-x_L, which acts similar to MCL-1 and also antagonizes BAX and BMF, could partially substitute for ERBB2 in antagonizing apoptosis. It would be helpful to validate this result of the NIH3T3 cell-based murine tumor model using epithelial breast cancer cells such as MCF-7. However, when we conditionally express ERBB2 in epithelial cells, they activate fail-safe mechanisms such as oncogene-induced senescence or apoptosis (13, 25), which first have to be bypassed before tumors could be formed. Therefore, systematic *in vivo* studies on interactions between ERBB2 and putative antiapoptotic factors in epithelial cells are very difficult to perform.

Our study confirms previous reports that ERBB2 influences the expression of Bcl-2 family members (6, 12). Moreover, ERBB2 expression correlated with the expression of several Bcl-2 family members in patients with breast cancer. However, because of the high interindividual variability, it would not be reasonable to routinely combine ERBB2-targeting therapy with a standard Bcl-2 family–antagonizing therapy. Rather, patients should be tested for overexpression of specific Bcl-2 members to allow an individualized targeting of the most relevant factors, such as *MCL-1*, *BMF*, *BAX*, or *BNIP3*.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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ERBB2 Induces an Antiapoptotic Expression Pattern of Bcl-2 Family Members in Node-Negative Breast Cancer

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