The Nuclear Transcription Factor κB/bcl-2 Pathway Correlates with Pathologic Complete Response to Doxorubicin-Based Neoadjuvant Chemotherapy in Human Breast Cancer

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Abstract

Purpose: Molecular factors involved in apoptosis may affect breast cancer response to chemotherapy. Herein, we studied the nuclear factor κB (NF-κB)/bcl-2 pathway to determine whether or not activation of this antiapoptotic pathway was associated with a poor response of human breast cancer to anthracycline-based neoadjuvant chemotherapy.

Experimental Design: We studied 82 human breast cancer samples from patients treated with neoadjuvant doxorubicin-based chemotherapy and studied whether or not nuclear location of the transcription factor NF-κB was associated with expression of bcl-2 and bax and whether or not expression of these proteins correlated with chemotherapy response. Protein expression was measured with immunohistochemical staining. A dedicated breast cancer pathologist who was unaware of the clinical outcome data dichotomized the slides as positive or negative based on the presence or absence of cytoplasmic staining for bcl-2 and bax or nuclear staining for NF-κB.

Results: Sixty-one percent of the tumors were positive for bcl-2, 85% were positive for bax, and 16% were positive for NF-κB. All bcl-2-positive tumors were also bax positive ($P < 0.0001$) and all NF-κB-positive tumors were both bcl-2 positive ($P = 0.001$) and bax positive ($P = 0.113$). Eleven of the 82 patients (13%) had a pathologic complete response (pCR) to chemotherapy. Patients with positive staining tumors for any of the markers less commonly achieved a pCR to chemotherapy than those with negative tumor staining. The pCR rates were NF-κB positive 0% (0 of 13) versus NF-κB negative 13% (11 of 69; $P = 0.130$); bcl-2 positive 4% (2 of 49) versus bcl-2 negative 27% (9 of 33; $P = 0.004$); and bax positive 6% (4 of 69) versus bax negative 58% (7 of 12; $P < 0.001$).

Conclusion: We conclude that nuclear localization of NF-κB correlates with bcl-2 and bax expression and that the NF-κB/bcl-2 pathway may be associated with a poor response to neoadjuvant doxorubicin-based chemotherapy.

Breast cancer response to chemotherapy is in part determined by the success of inducing apoptosis in tumor cells. Our group and others have shown a correlation between the degree of treatment-induced apoptosis and the response of human breast cancer to neoadjuvant chemotherapy (1, 2). Several other investigators have indirectly explored the relationship between apoptosis and chemotherapy response by evaluating the correlation with the expression of proteins involved in the apoptotic pathway, such a p53, bcl-2, and bax (3–12). In general, such studies have yielded mixed results, in part because apoptosis results from a complex interplay of a number of proteins and expression of a single marker may not provide a complete understanding of the involvement of the apoptotic pathway. Another frequent limitation of such studies is that they evaluated patients with various disease stages who were treated with a variety of different chemotherapy regimens.

The primary objective of this study was to investigate how tumor cell expression of bcl-2 and bax correlated with response to neoadjuvant 5-fluorouracil, doxorubicin, cyclophosphamide (FAC) chemotherapy. To minimize confounding biases, we studied only patients treated on two prospective clinical trials that involved FAC chemotherapy before surgery. We hypothesized that expression of the antiapoptotic protein bcl-2 would be associated with a poor chemotherapy response and expression of the proapoptotic protein bax would be associated with the achievement of a pathologic complete response (pCR). After finding that expression of both factors correlated with a poor response, we

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further hypothesized that a transcription factor involved in the regulation of both protein products may be playing a significant role in controlling apoptotic response. Nuclear factor κB (NF-κB) is a nuclear transcription regulator with a specific motif for bcl-2 transcription (13). Recently, NF-κB has also been shown to be positively associated with bax expression (14, 15). Correspondingly, we studied whether or not nuclear location of the NF-κB protein correlated with bcl-2 and bax expression and investigated how the NF-κB/bcl-2 pathway correlated with response to treatment.

Materials and Methods

This study used tumor material obtained from paraffin blocks of the pretreatment biopsy specimens from patients who participated on two sequential Surveillance Committee (Institutional Review Board)–approved clinical trials conducted at the University of Texas M.D. Anderson Cancer Center between the years 1989 and 1993. The Institutional Review Board also approved the use of the archived material and the abstraction of clinical data from patient medical records for the specific purpose of this study.

Both of the trials investigated neoadjuvant FAC chemotherapy for patients with stage II or III breast cancer. Three hundred seventy-two patients were enrolled in these studies. Two hundred forty-four of these were diagnosed with a fine needle aspiration before chemotherapy and therefore did not have tissue stored. We attempted to obtain pretreatment tissue blocks from the other 134 cases and received suitable pretreatment tissue in 82 cases. The material from these patients has been used in other investigations (16, 17). Clinical features and outcome of the 82 studied cases were not significantly different than the features and outcome of the entire group enrolled on these protocols.

Per protocol, all patients were treated with four cycles of neoadjuvant FAC chemotherapy before surgery. Sixty patients were treated according to the following dose schedule of FAC: 500, 50, and 500 mg/m^2, respectively. The remaining 22 cases received FAC with the following dose schedule: 600, 60, and 1,000 mg/m^2, respectively. This dose-escalated schedule of FAC was found to be equivalent to the standard FAC schedule in terms of clinical outcome (18).

Immunostaining. Slides for immunohistochemistry staining were made from 4-nm tissue sections taken from paraffin blocks that were mounted on charged slides. Slides were then deparaffinized and rehydrated with descending grades of ethyl alcohol. Immunohistochemistry staining was done using the following antibodies: bcl-2 (diluted 1:250), bax (diluted 1:200), and NF-κB (diluted 1:100; all from Santa Cruz Biotechnology, Santa Cruz, CA). Only materials from biopsy specimens taken before chemotherapy treatment were analyzed. All stainings were done with positive and negative controls.

A breast cancer pathologist (A.A.S.) interpreted the slides without knowledge of the clinical outcome of each case. Bcl-2 and bax positivities were defined by the presence of any cytoplasmic staining of the tumor cell cytoplasm. NF-κB positivity was defined as greater staining of protein within the nucleus compared with the cytoplasm.

HER2/neu expression, mitosin immunohistochemistry staining, nuclear grade, and epidermal growth factor receptor (EGFR) immunohistochemistry were analyzed in previous studies (1, 15). HER2/neu expression was considered positive if there was 3+ positive immunostaining. Tumors with 1+ or 2+ staining were analyzed with fluorescence *in situ* hybridization and considered positive if there were more than two gene copy numbers within a cell. Mitosin staining was considered high if there were >6 mitotic figures per 10 high-power fields and considered low if there were ≤6. EGFR was considered positive for any tumor that had membranous staining. Additional details of HER2/neu, mitosin, and EGFR staining and interpretation were provided in our earlier reports (16, 17).

Statistical analysis. A χ^2 or Fisher's exact test was used to analyze the relationships of biomarkers with each other and how these markers correlated with pCR rates. pCR was defined as no residual invasive breast cancer in both the breast surgical specimen and the resected axillary lymph nodes. The Kaplan-Meier method was used to analyze the relationship of bcl-2, bax, and NF-κB with overall survival. For these analyses, the date of diagnosis was used as time 0. Differences in survival curves were assessed with the log-rank test using two-sided P values. We did not perform a Cox regression analysis because the number of patients who achieved a pCR was too small for a Cox analysis to have validity.

Results

The median age of the study patients at the time of diagnosis was 46 years. Thirty-three percent of patients had clinical stage II (1988 American Joint Committee on Cancer staging system) breast cancer at diagnosis, 63% had clinical stage III disease, and 4% had stage IV disease (supraclavicular lymph node involvement without systemic metastases). After chemotherapy, 58 patients underwent a modified radical mastectomy and 24 patients had a breast conserving surgery.

Bcl-2 staining was positive in 60% of the cases (49 of 82) and negative in 40% (33 of 82). Bax staining was positive in 84% of the cases (69 of 82), negative in 15% (12 of 82), and one case was inconclusive. Finally, nuclear staining of NF-κB was present in 13% (12 of 82) of the tumors whereas 83% (67 of 82) had a predominant cytoplasmic staining pattern. Staining for NF-κB was inconclusive in the three remaining cases. Figure 1 shows an example of a NF-κB-positive tumor and a NF-κB-negative tumor. There was no correlation of bax, bcl-2, or NF-κB expression and clinical stage or patient age.

All 49 bcl-2-positive tumors were also bax positive ($P < 0.0001$). Furthermore, all 11 NF-κB-positive tumors were both bcl-2 and bax positive ($P < 0.0001$). The relationships of these markers to other pathologic tumor features are highlighted in Tables 1–3. Bcl-2-positive, bax-positive, and NF-κB-positive tumors were more often of low or intermediate nuclear grade and had a lower proliferative index, as defined by a mitosin staining of ≤15%, than tumors that were negative for these markers.

Eleven of the 82 patients (13%) had a pCR to chemotherapy. Bcl-2 expression was inversely correlated with pCR. Rates of pCR were 4% in patients with bcl-2-positive tumors versus 27% in patients with bcl-2 negative tumors ($P = 0.004$). The rate of pCR was also lower in the patients with bax-positive tumors compared to patients with bax-negative tumors (6% versus 58%, respectively; $P < 0.0001$). Finally, none of the patients with nuclear location of NF-κB achieved a pCR compared with a rate of 14% (11 of 79) in those with NF-κB located in the cytoplasm ($P = 0.134$). Table 4 shows the rates of pCR according to the combination of all three of these biomarkers. Interestingly, the respective pCR rate for the 12 tumors that were positive for all three markers was 0% and the pCR rate for the 12 tumors that were negative for all three markers was 58%. We also analyzed the correlation of these markers with the percentage of cases with no residual invasive disease in the breast. Using this broader definition of pCR, 24 of the 82 cases achieved a pCR. The respective rates of pCR for positive and negative cases were not statistically different for bcl-2 ($P = 0.804$) or NF-κB ($P = 0.288$) but continued to be different for bax ($P = 0.032$).

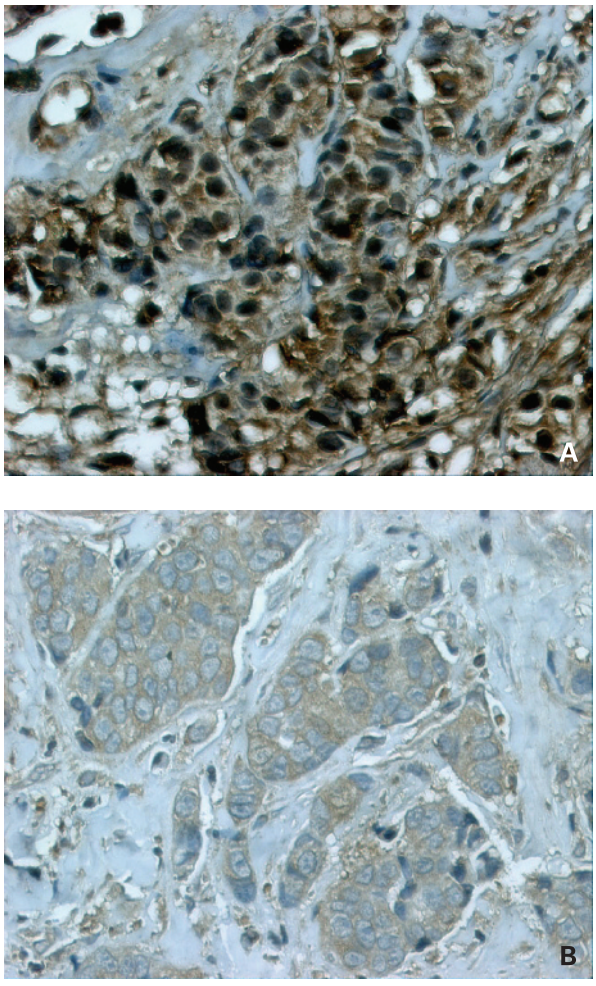

Fig. 1. An example of a NF-κB-positive tumor (*A*) and a NF-κB-negative tumor (*B*). Positivity was determined by a greater degree of nuclear staining than cytoplasmic staining.

The 10-year overall survival for the population was 57%. There was no statistical correlation between expression of bcl-2, bax, or NF-κB and overall survival. The respective 10-year rates for positive and negative staining were as follows: bcl-2, 56% versus 48% ($P = 0.579$); bax, 54% versus 56% ($P = 0.587$); and NF-κB, 38% versus 61% ($P = 0.979$). In addition, the expression of the three markers did not correlate with disease-free survival (bcl-2, $P = 0.190$; bax, $P = 0.454$; NF-κB, $P = 0.395$) or local-regional recurrence-free survival (bcl-2, $P = 0.187$; bax, $P = 0.102$; NF-κB, $P = 0.951$). Given the size of the respective subgroups, the statistical power to detect survival differences was low.

Discussion

In this study, we showed that the nuclear localization of NF-κB was associated with expression of bcl-2 and bax and that activation of the NF-κB/bcl-2 pathway may be a mechanism for tumor resistance to anthracycline-based chemotherapy. Whereas previous studies have correlated the expression of bcl-2 and bax with response of breast cancer to chemotherapy, this is the first study to investigate the importance of NF-κB in these relationships. We found that none of the cases with activation of NF-κB achieved a pCR to neoadjuvant FAC treatment and, conversely, we also noted a high rate of pCR (58%) in the tumors that were negative for all three of the biomarkers.

NF-κB is a transcription factor involved in the regulation of important signaling pathways, including those related to the apoptotic response of cells to injury and cytotoxic treatments. When not activated, NF-κB exists in the cytoplasm of cells as a p65/p50 heterodimer that is retained in its inactive state by its association with IκB, an inhibitory protein (19). Activation of this complex results in phosphorylation and subsequent dissociation of IκB, permitting nuclear translocation of the freed NF-κB protein, where it then regulates transcription. NF-κB is a direct transcription regulator of bcl-2 (13). Our finding that all NF-κB-positive tumors were also bcl-2 positive is consistent with this known relationship. We hypothesize that activation of the NF-κB/bcl-2 pathway leads to inhibition of chemotherapy-induced apoptosis, which results in treatment resistance. This hypothesis is also supported by previously published data from preclinical models that discovered that activation of NF-κB inhibits chemotherapy-induced apoptosis (20, 21).

Table 1. Bcl-2 staining and selected pathologic characteristics

Characteristic	Bcl-2 positive ($n = 49$)	Bcl-2 negative ($n = 33$)	*P*
Bax			
Negative	0	12	<0.0001
Positive	49	20	
Inconclusive		1	
NF-κB			
Negative	35	32	0.002
Positive	12	0	
Inconclusive	2	1	
EGFR			
Negative	39	24	0.745
Positive	8	6	
Unknown	2	3	
HER2/neu			
Negative	38	26	0.896
Positive	11	7	
Modified Black's nuclear grade			
G1	4	1	0.059
G2	19	6	
G3	26	26	
Estrogen receptor			
Negative	26	21	0.349
Positive	23	12	
Progesterone receptor			
Negative	23	19	0.351
Positive	26	14	
Mitosin			
<15%	34	12	0.003
>15%	15	21	

Table 2. Bax staining and selected pathologic characteristics

Characteristic	Bax positive (n = 69)	Bax negative (n = 12)	P
Bcl-2			
Negative	20	12	<0.0001
Positive	49	0	
NF-κB			
Negative	54	12	0.111
Positive	12	0	
Inconclusive	3		
EGFR			
Negative	54	8	0.892
Positive	12	2	
Unknown	3	2	
HER2/neu			
Negative	53	10	0.621
Positive	16	2	
Modified Black's nuclear grade			
G1	5	0	0.081
G2	24	1	
G3	40	11	
Estrogen receptor			
Negative	39	7	0.908
Positive	30	5	
Progesterone receptor			
Negative	35	6	0.964
Positive	34	6	
Mitosin			
<15%	43	3	0.016
>15%	26	9	

Moreover, we also found that NF-κB activation also correlates with expression of bax. Whereas NF-κB has not be shown to directly regulate transcription of bax, NF-κB does directly regulate the transcription of the ataxia telangiectasia mutated (*ATM*) gene (22, 23), which in turn can regulate transcription of p53. It is possible that expression of p53 can then lead to proapoptotic signaling and expression of bax. Indeed, a recent study investigating human glioma samples found that nuclear localization of NF-κB correlated with bax expression, similar to the results presented in this study (14). However, other data have suggested that inhibition of NF-κB can increase bax levels, a finding contradictory to the results of our study (15).

We found that nuclear localization of NF-κB, bcl-2 expression, and bax expression were correlated with lower-grade disease and lower rates of proliferation. It is possible that the effects of NF-κB on the cell cycle may have influenced this finding. For example, investigators have found that NF-κB activation inhibits proliferation through induction of the cell cycle inhibitor p21 (24). However, the data about the cell cycle effects of NF-κB activation are also mixed, in that NF-κB can increase proliferation through transcription of cyclins D1, D2, D3, and E (25–28).

To date, there are few data available that correlate NF-κB expression with downstream signaling and clinical outcome in human tumors. In one of the only other human studies, investigators found that NF-κB activation in esophageal adenocarcinoma correlated with a poor response to neoadjuvant chemoradiation treatments (29). Specifically, the pCR rate after chemoradiation was 60% (12 of 20) in patients with NF-κB-negative tumors versus a rate of 3% (1 of 38) in patients with NF-κB-positive tumors.

Whereas limited data have been published about NF-κB, others have previously investigated how bcl-2 and bax expression correlates with response to anthracycline chemotherapy in human breast cancer. For example, in a study of 104 patients treated with an anthracycline/docetaxel–based chemotherapy regimen before surgery, a lack of bcl-2 expression correlated with a better pathologic response (although this study did not define a favorable pathologic outcome by achievement of pCR; ref. 30). In addition, an earlier study of 55 patients with locally advanced disease treated with neoadjuvant chemotherapy (some of whom were treated with an anthracycline) also found a similar correlation between bcl-2 expression and a poor response to treatment (31). However, other authors have not found this relationship (32). Interpretation of these discordant results is difficult because oftentimes such studies have included patients treated with a variety of disease stages and a variety of chemotherapy regimens. One of the strengths of our study was that all patients had similar stage

Table 3. NF-κB staining and selected pathologic characteristics

Characteristic	NF-κB positive (n = 12)	NF-κB negative (n = 67)	P
Bax			
Negative	0	12	0.111
Positive	12	54	
Inconclusive		1	
Bcl-2			
Negative	0	32	0.002
Positive	12	35	
EGFR			
Negative	10	50	0.374
Positive	1	13	
Unknown	1	4	
HER2/neu			
Negative	8	53	0.351
Positive	4	14	
Modified Black's nuclear grade			
G1	0	3	0.088
G2	7	18	
G3	5	46	
Estrogen receptor			
Negative	4	40	0.093
Positive	8	27	
Progesterone receptor			
Negative	3	37	0.055
Positive	9	30	
Mitosin			
<15%	12	32	0.001
>15%	0	35	

Table 4. Staining patterns and pCR rate

	*n**	pCR rate
NF-κB positive, bcl-2 positive, bax positive	12	0% (0 of 0)
NF-κB negative, bcl-2 positive, bax positive	35	6% (2 of 35)
NF-κB negative, bcl-2 negative, bax positive	19	11% (2 of 19)
NF-κB negative, bcl-2 negative, bax negative	12	58% (7 of 12)

*Total number <82 due to three inconclusive NF-κB readings and one inconclusive bax reading.

disease and received four cycles of protocol-specified neoadjuvant chemotherapy.

Our finding that no patients with nuclear localization of NF-κB achieved pCR may have therapeutic as well as predictive implications. If validation studies confirm that the NF-κB/bcl-2 pathway is associated with resistance to anthracycline chemotherapy, this pathway may represent a new therapeutic target. Specifically, our group is currently investigating combining bcl-2 antisense therapy with chemotherapy for patients with locally advanced breast cancer. In addition, a number of NF-κB inhibitors are in clinical development, and studying combinations of these inhibitors with chemotherapy in breast cancer patients who display a nuclear localization of NF-κB would be justified.

In conclusion, our data indicate that nuclear localization of NF-κB strongly correlates with bcl-2 and bax expression and that this signaling pathway may be one determinant of resistance to anthracycline chemotherapy in human breast cancer. Given the potential importance of this relationship, these data warrant additional studies investigating the NF-κB/bcl-2 pathway and chemotherapy response.

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