PROGNOSTIC SIGNIFICANCE OF APOPTOSIS RELATED GENE FAMILY bcl-2 IN HUMAN BREAST CANCER

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ABSTRACT

Objective: To study the prognostic effect of bcl-2 oncogene and its gene family members bax, bcl-x expression in breast cancer patients. Methods: Expression of bcl-2, bax proteins in 91 human breast cancer tissue sections were studied by immunohistochemical method. bcl-x1 mRNA expression in frozen tissues from 16 breast cancer patients were detected using Northern blot method. Results: bcl-2 protein positivity was found in 60/91 (65.9%) patients, and bax positivity 59/91 (64.8%). bcl-2 and bax expression levels were associated with apoptotic index(AI), histological grade, axillary lymph node metastasis, postoperative local recurrence and metastasis. bcl-2 expression was related to ER positivity. In univariate analysis for disease free survival (DFS), bcl-2 and bax protein levels, and Al were all found to have prognostic value. The result of Cox's model multivariate analysis showed that bcl-2 protein level was an independent prognostic factor. In 16 frozen breast cancer tissues, 8/16(50%) had higher level of bcl-x1 mRNA, which showed correlation with bcl-2 protein expression and axillary lymph node metastasis. Conclusion: The findings indicate that dysregulated expressions of bcl-2, bax and bcl-x1 apoptosis-related genes, suggestive of serious deregulation of apoptotic process, may contribute to the biologic aggressiveness of breast cancer. bcl-2 protein is an independent indicator of prognosis in breast cancer patients.

Key words: Breast cancer, Prognosis, Apoptosis, Oncogene

Received August 26, 1999; accepted December 21, 1999

This work was supported by the National Natural Science Foundation of China (No. 39670803).

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Bcl-2 family proteins appear to regulate apoptosis, with bcl-2, bcl-x1 function as suppressors of apoptosis and bax, bcl-xs as promoters of cell death.[1] The prognostic significance of these proteins in breast cancer has been reported, but a comprehensive study is needed. In the present study, we have investigated the expression of bcl-2, bax, bcl-x1 in this multigene family, as regards to their prognostic value in breast cancer.

MATERIALS AND METHODS

Patients

Ninety-one breast cancer patients who had undergone surgery at our hospital from 1981 to 1990 were studied. The median age was 50 years. They were all pathologically evaluated to be invasive breast cancer, 85 cases were non-specific type and 6 cases were specific type. There were 45 cases (49.5%) with metastasis in the axillary lymph nodes, and 46 cases (50.5%) with no metastasis. Estrogen receptor (ER) positive was found in 56 cases, ER negative in 35 cases. In view of TNM stage, 18 patients (19.8%) belong to stage I, 50 cases (54.9%) were stage II, and 23 cases (25.3%) were stage III. During the period of observation, 10 patients died of the disease, 23 had local recurrences or distant metastasis.

Fresh Tissues

Sixteen breast cancer patients had undergone surgery in our hospital from 1996 to 1997 and their fresh tumor tissues were stored in liquid nitrogen.

Immunohistochemical Agents

The monoclonal antibody to bcl-2 (Clone 100) and polyclonal antibody to bax (N-20) were obtained from

Santa Cruz Biotechnology Inc. USA. The working concentration of bcl-2 was 1:100, and that of bax was 1:40. Immunostaining utilized LSAB+ kit of DAKO, Denmark (K0679, biotinylated IgG-streptavidin horseradish peroxidase). 3, 3-diaminobenzidine tetrahydro-(DAB) was also obtained from DAKO.

Other Agents

Human breast cancer cell line MCF-7, bcl-x1 cDNA probe, and 18s probe were given as a present from Dr. Fontana (Cancer Center, University of Maryland, USA). *In Situ* cell death detection kit (POD) was obtained from Boehringer Mannheim Inc. (Cat No. 1684817), Germany. Total RNA extraction reagent (TRIZOL) was from GIBCO, USA. α-^{32}P-dCTP(111TBq/mmol) and random primer label kit were purchased from Amersham, UK.

Labelling of probes and hybridization were performed at Isotope Laboritory, Shanghai Cancer Institute.

Immunohistochemistry

LSAB method was utilized. After deparaffinization and rehydration, the sections were digested in 0.05% trypsin at 37°C for 30 min. Then the slides were incubated for 15 min with 0.3% H_2O_2 to block endogenous peroxidase at room temperature. The sections were then incubated overnight at 4°C with bcl-2 and bax antibody. After rinsing with PBS, sections were covered with biotinylated IgG from DAKO Lab. kit and incubated at 37°C for 20 min. Then slides were rinsed with PBS, incubated with streptavidin horseradish peroxidase for 30 min, and washed with PBS. The colorimetric detection was performed by incubation of the specimens in 0.04% DAB + 0.03% H_2O_2 at room temperature. Sections were finally counter-stained with hematoxylin, cleared and mounted.

Immunostaining Criteria

The immunohistochemical positive cells referred to as cytoplasmic staining or plasma membrane and nuclear membrane stained cells. The percentage of immunopositive cells were determined by counting 1000 tumor cells in 10 randomized high-powered fields (×400): 0–10% was defined as (-), 10%–50% as (+), and >50% as (++).

For both bcl-2 and bax, sections from normal lymph nodes were used as positive control and tumor sections in which primary antibody was omitted served as negative control.

TUNEL Labeling and AI Counting

It was performed as described previously.[21] Bloom and Richardson histologic grading was done with reference to Elston and Ellis modified method.[3]

Northern Blot

Total RNA was extracted by using the modified Chomozynski and Sacchi one-step method.[4] The total RNA of MCF-7 cell was utilized as positive control. RNA was stored at -70°C. Same amount of samples was electrophorased on a 1.2% agarose formadehyde gel. Under UV light, degradation of the extract RNA was not found. Bands of 18S and 28s were clear, and photos were taken. Then RNA was transferred onto nitrocellulous membrane, and cross-linked in a vacuum. ^{32}P-labeled bcl-x1 probe and 18S probe were prepared by random primer method. Then hybridization and autoradiography were undertaken. Band intensity was analyzed by using an image analysis system, and calibrated by comparison with the 18S bands.

Followed-up

From Dec 19 to 22, 1996, we checked patient histories in our out patient department and defined the latest interview as the last follow-up date. The median follow-up time was 5 years.

Statistical Methods

Statistical analysis was performed using the SPSS system at Department of Epidemiology, Shanghai Cancer Institute. Spearmen's rank-based correlation was used to assess the relationship between the variables. Univariate analysis of RFS and overall survival (OS) were performed by the method of Kaplan-Meier, log-rank test. Survival curves were drawn according to life table. Cox's proportional hazards model was used to analyze the multivariate factors of survival rate.

RESULTS

Expression of bcl-2, bax Protein in Breast Cancer Tissues

The immunohistochemical staining patterns for bcl-2 and bax showed characteristic cellular localization in the cytoplasm of cancer cells. In some tumor cells a perinuclear intensification of staining was observed. Both proteins expressed heterogeneously in breast cancer. 60/91 (65.9%) carcinomas showed positive immunoreaction for bcl-2 and 59/91 (64.8%) for bax. 27/91 (29.7%) carcinomas were considered bcl-2 high expressors, and 28/91 (30.8%) were high expressors for bax (Figure 1).

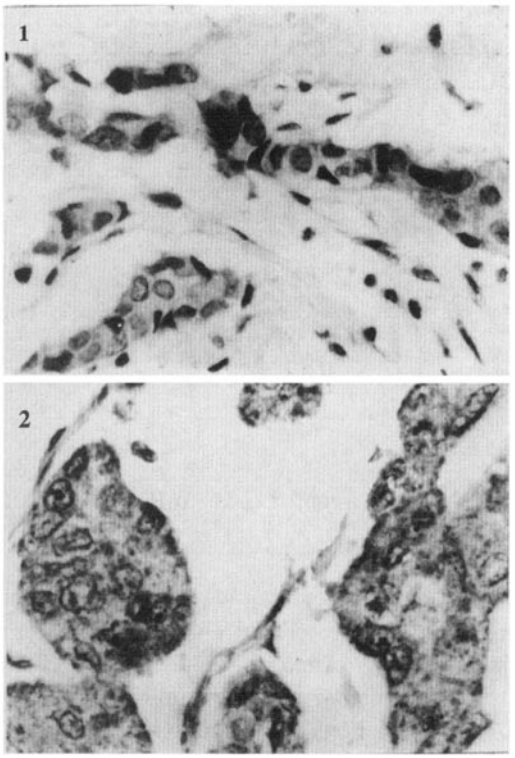

Fig. 1. High bcl-2 (1) and bax (2) immunoreactivity in breast infiltrating ductal carcinoma sample. (×400)

Relationship among bcl-2, bax and Clinical Pathological Factors in Breast Cancer

There was a significant correlation between bcl-2 expression and poor histological grade, lymph node metastasis and ER status. In contrast to bcl-2, we found an inverse relationship between bax immunoreactivity and poor tumor grade and lymph node metastasis. High bcl-2 and low bax were found to be associated with post-operative local recurrence and metastasis.

Relationship among bcl-2, bax and AI in Breast Carcinomas

A significant correlation was seen between high bcl-2 and low bax (Rs=0.564, $P<0.001$). Our results also demonstrated that high Al was significantly related to low bcl-2 (Rs=0.699, $P<0.00$ 1), and high bax (Rs=0.568, $P<0.001$) in breast cancer tissues.

Survival Analysis of bcl-2 and bax

Univariate survival analysis: The variables associated with disease free survival (DFS) were tumor grade, axillary lymph node metastasis, AI, bax and bcl-2 (Table 1).

Multivariate survival analysis: In multivariate analysis of our data, bcl-2 expression ($P=0.0046$) and tumor grade ($P=0.0213$) were proved to be the indepen-dent prognostic markers for DFS.

Correlation of bcl-2/bax, bcl-2/AI and bax/AI Phenotypes with Survival

In these various phenotypes of breast carcinomas, bcl-2(H)/AI(L), bax(L)/AI(L) and bcl-2(H)/bax(L) had the worst prognosis in their own group, which resulted in 75%, 43.5% and 76% of the patients having relapsed or died (Table 2).

Kaplan-Meier Survival Curves

The patients who had tumors with low bcl-2 and high bax immunoreactivity survived longer without recurrence than those with low bax ($x^2=9.82$, $P=0.0017$) and high bcl-2 ($x^2=54.09$, $P=0.0000$) (Figure 2).

Table 1. Univariate survival analysis for prognostic factors in this group of patients (log-rank test)

Variables	Relapse free survival		
	x^2	DF	P
Histologic type	0.96	1	0.3264
Grade	37.78	1	0.0000**
Invasion	19.07	1	0.0000**
Axillary lymph node metastasis	29.95	1	0.0000**
Diameter of the tumor mass	10.43	1	0.0012*
ER	0.03	1	0.8527
AI	6.21	1	0.0127*
bcl-2 (L, H)	54.09	1	0.0000**
bax (L, H)	9.82	1	0.0017*

*$P<0.05$, **$P<0.001$, DF: Degree of freedom

Table 2. The patient survival in different phenotypes of bcl-2/AI, bcl-2/bax, and bax/AI expression

Phenotype	No. of patients	Healthy	Relapsed	Death
bcl-2/AI				
bcl-2(H) AI(L)	24	6 (25%)	10 (41.7%)	8 (33.3%)
bcl-2(L) AI(H)	32	30 (93.8%)	1 (3.1%)	1 (3.1%)
bax/AI				
bax(H) AI(H)	18	17 (94.4%)	1 (5.6%)	0 (0)
bax(L) AI(L)	46	26 (56.5%)	11 (23.9%)	9 (19.6%)
bcl-2/ bax				
bcl-2(H) bax(L)	25	6 (24%)	11 (44%)	8 (32%)
bcl-2(L) bax(H)	26	26 (100%)	0 (0)	0 (0)

$P<0.05$; H: High expression; L: Low expression

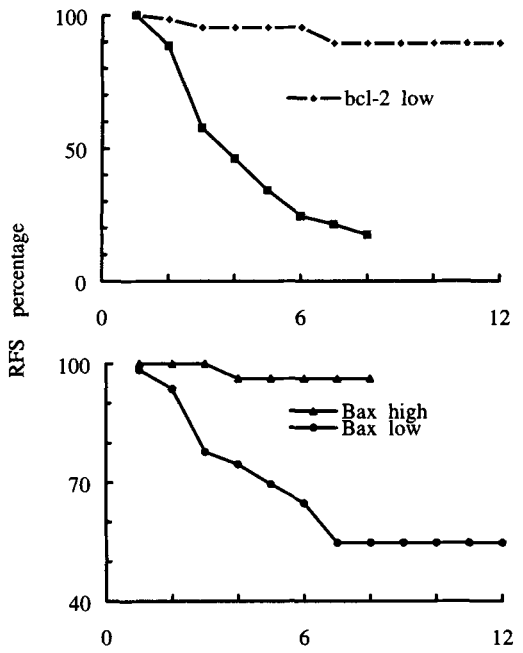

Fig. 2. Relapse free survival curves in breast cancer patients with low and high levels of bcl-2 and bax protein

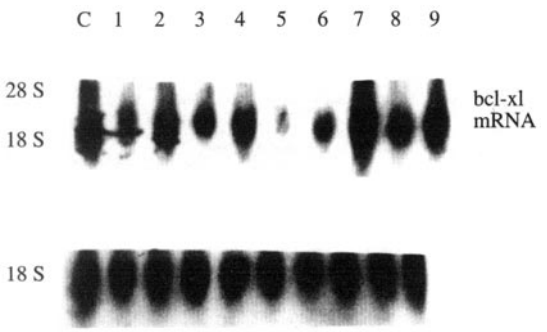

Fig. 3. bcl-x1 mRNA expression in breast carcinoma tissues and human breast cancer cell line. 1– 9: breast carcinoma tissues C: human breast cancer cell line MCF-7 as a control

Relationship between bcl-xl mRNA and Tumor Characteristics

Based on the Spearmen correlation test, our results demonstrated that bcl-x1 mRNA expression (Figure 3) was significantly related to bcl-2 protein (Rs=0.579, P=0.029) and axillary lymph node metastasis (Rs=0.524, P=0.033). Correlations among bcl-x1 mRNA and tumor grade, ER, tumor size, AI, bax were not statistically significant.

DISCUSSION

Using immunohistochemical staining, we investigated the expression of bcl-2 and bax protein in breast carcinomas, and found that immunostaining patterns for bcl-2 and bax showed cytoplasmic localization, with perinuclear intensification in few tumor cells. The positive rate was 65.9% for bcl-2 and 64.8% for bax. Our results could be reconciled with other reports.[5,6] In our study, we demonstrated that in bcl-2-high, bax-low breast cancers the Al was significantly lower than it in bcl-2-low, bax-high cancers. In agreement with the theory that bax acts as an accelerator of apoptosis, opposing bcl-2 effects cell life. We also found that high bcl-2 inimunoreactivity was correlated positively with high tumor grade, presence of axillary lymph node metastasis, post-operative local recurrence and metastasis. In contrast, we found an inverse correlation between bax expression and tumor grade, lymph node involvement, TNM stage, post-operative recurrence and metastasis. These results illustrated that apoptosis related gene investigation was helpful for evaluation of breast carcinoma differentiation. Bukholm et al.[7] investigated the relationship between bcl-2 and $p21^{WAF1/CIP1}$, and found that in breast carcinomas with wild-type p53, high bcl-2 could inhibit $p21^{WAF1/CIP1}$ expressions. Since $p21^{WAF1/CIP1}$ plays important role in G_1 checkpoint of cell cycle, bcl-2 might interfere with the function of p53, resulting in tumor progression.

Lipponan[6] demonstrated in a group of breast cancer patients, bcl-2 in T_1N_1 breast carcinomas was higher than that in T_1N_0 tumors. In multivariate analysis, he also demonstrated that in a group of PR positive, well-differentiated T_1 breast carcinomas, significant correlation existed between bcl-2 and axillary lymph node involvement.

In univariate analysis for prognostic factors affecting breast patients' survival rate, bcl-2, bax and AI were demonstrated to be significant, apart from tumor grade, tumor size and axillary lymph node metastasis. Our study further showed bcl-2 was an independent prognostic marker in multivariate analysis. Most studies investigated prognostic value of bcl-2 by univariate analysis, and bcl-2 was not proved to be an independent marker in multivariate analysis.[5] Few studies reported prognostic value of bax in breast carcinoma. In a group of metastatic breast cancer patients, low bax patients showed a significantly poorer response to therapy and shorter survival in multivariate analysis.[8] Kapranos[9] demonstrated in a series of lymph node negative breast cancer patients, cases with no bax immunoreactivity were prone to have metastasis. In the present study, we did not show AI and bax to be of independent prognostic value. The reason might be that close correlation of apoptosis regulatory factors was present in Cox's model, and only bcl-2 was demonstrated to be of independent value. We don't think the prognostic value of Al and bax should be ignored. We further investigated the coexpression status of bcl-2/bax, bcl-2/AI and bax/AI in relation to clinical outcome. It was found that bcl-2(H)/bax(L), bcl-2(H)/AI(L) and bax(L)/AI(L) each correlated with poor prognosis. The relapse and cases which died accounted for 75%, 43.5% and 76% in each status. The knowledge of the status of both bcl-2 and bax in relation to AI, provides more accurate information about prognosis of breast cancer.

bcl-x1 is one of the two types of complementary DNA of bcl-x. It acts as a suppressor of apoptosis. bcl-x1 gene expression in breast cancer tissues has not been investigated extensively. Using Northern blot, we studied bcl-x1 mRNA expressions in 16 fresh breast carcinoma specimens in relation to clinical and pathological features, and found a positive correlation between bcl-x1 mRNA and axillary lymph node metastasis, bcl-2 protein expression. By immunostaining, Olopade[10] investigated bcl-x1 protein expression in invasive breast carcinomas, and found high bcl-x1 cases made up 43%. It was also demonstrated that bcl-x1 expression was positively related to high tumor grade, lymph node metastasis and lower overall survival rate. These studies indicated that bcl-2 family proteins appear to regulate apoptosis in breast carcinoma. Inhibition of apoptosis is expected to contribute to accelarated proliferation. Disturbances in regulation of apoptosis may result in metastatic lymph node dissemination and poor prognosis of breast carcinoma.

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