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BCL-2 AND BAX EXPRESSION AND PROSTATE CANCER OUTCOME IN MEN TREATED WITH RADIOTHERAPY IN RADIATION THERAPY ONCOLOGY GROUP PROTOCOL 86–10

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Abstract

Purpose—Bcl-2 and bax are proteins with opposing roles in apoptosis regulation; yet abnormal expression of either has been associated with failure after radiotherapy (RT). In this study we examined bcl-2 and bax expression as predictive markers in men treated with radiotherapy ± androgen deprivation on Radiation Therapy Oncology Group (RTOG) protocol 86-10.

Experimental Design—Suitable archival diagnostic tissue was obtained from 119 (26%) patients for bcl-2 analysis and 104 (23%) patients for bax analysis. Cox proportional hazards multivariate analysis was used to determine the relationship of abnormal bcl-2 and bax expression to the end points of local failure, distant metastasis, cause-specific mortality, and overall mortality. Bcl-2 overexpression was classified as any tumor cell cytoplasmic staining and altered bax expression was classified as greater or lesser cytoplasmic staining intensity of tumor cells as compared with adjacent normal prostate epithelium.

Results—The study cohort exhibited bcl-2 overexpression in 26% ($n = 30$) of cases and abnormal bax expression in 47% ($n = 49$) of cases. A borderline significant relationship was observed between abnormal bax expression and higher Gleason score ($p = 0.08$). In univariate and multivariate analyses, there was no statistically significant relationship seen between abnormal bcl-2 or bax expression and outcome.

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Conclusions—Abnormal bcl-2 and bax expression were not related to any of the end points tested. The cohort examined was comprised of patients with locally advanced disease and it is possible that these markers may be of greater value in men with earlier-stage prostate cancer.

Keywords

Bcl-2; Bax; Androgen Deprivation; Radiation Therapy

INTRODUCTION

Bcl-2 and its family proteins exercise control on cell response to genotoxic stress (1). The protooncogene product bcl-2 heterodimerizes with its pro-apoptotic relative bax (2), dictating in part the sensitivity or resistance of cells to apoptotic stimuli, including radiation (RT). Previous studies have established bcl-2 and bax proteins as candidate immunohistochemical markers of prostate cancer outcome in response to external beam radiotherapy (3–7) and brachytherapy (8). In most of these studies abnormal bcl-2 and bax expression was associated with a higher risk of treatment failure. However, Bylund *et al.* (3) found abnormal bcl-2 expression to be related to a favorable outcome in their cohort of prostate cancer patients treated with radiotherapy (RT).

Bcl-2 is also implicated in the progression of prostate tumors to androgen-independence (9). Its expression is increased after androgen deprivation (9–12) and high levels are maintained in patients who fail androgen deprivation therapy (13,14).

The current analysis is of men enrolled in Radiation Therapy Oncology Group (RTOG) protocol 86-10, a phase III randomized trial comparing RT alone with RT plus short-term neoadjuvant and concurrent androgen deprivation (STAD) (15). The patients had locally advanced prostate carcinoma. These are the first analyses of the relationships of bcl-2 and bax expression to outcome in men treated on a large multi-institutional trial, and the first to investigate such relationships in the setting of RT+STAD.

METHODS AND MATERIALS

Study population

The study population was derived from a subset of patients enrolled in RTOG protocol 86–10 (15). Pretreatment tissue blocks were sectioned, stained, and reviewed for presence of tumor. For the bcl-2 analysis, 119 tissue samples had sufficient tumor and staining found suitable for analysis. Of these, 86 (72%) were from patients less than 75 years of age at time of enrollment (Table 1). Gleason score (GLSC) 7–10 was seen in 87 patients (74%; 1 patient was missing a Gleason score). The distribution by clinical T-category was 33 with T2 and 86 with T3 disease. Sixty-seven and 52 patients were assigned to RT alone and RT+STAD, respectively.

In the bax analysis, 82 of 104 patients analyzed were less than 75 years of age. Gleason Score 7–10 was seen in 76 (74%; 1 patient was missing a Gleason score). The distribution by clinical T-category was 24 patients with T2 and 80 with T3 disease. Fifty-seven patients were assigned to RT alone and 47 patients underwent RT+STAD.

Immunohistochemical technique

The paraffin-embedded formalin-fixed pretreatment diagnostic tissue blocks were cut 4 μm thick onto poly L-lysine slides and heated for 10 min in an 85 to 90°C oven. The tissue was then deparaffinized in xylene, rinsed well over several ethanol washes (95%) and finally with distilled water. The slides were then pressure-cooked in citrate buffer for 50 min for antigen retrieval. Following a rinse with water, 3% hydrogen peroxide was applied to the slides for 5

min at room temperature. Another rinse with Tris Buffer was performed before the primary bcl-2 (Cat. no. M0887, clone 124, Dako Corporation [Carpinteria, CA]; 1:100 dilution) or bax (Cat. no. 18–0218, clone 2D2, Zymed Laboratories Inc. [South San Francisco, CA]; 1:200 dilution) antibodies were overlaid. The slides were again rinsed in Tris Buffer twice, then incubated with Biotin (Dako LSAB II Kit) for 10 min, rinsed again as before, and incubated with Streptavidin (10 min). After a final rinse with Tris buffer, chromagen (DAB, Research Genetics, Huntsville, AL) was applied for 5 min. Counterstaining was then performed with commercially prepared hematoxylin (Dako Corporation, Carpinteria, CA) for 5 min, after which the slides were dehydrated and coverslipped with Permount. A Dako Autostainer was used for all staining.

Two investigators (R.L., T.J.M.) reviewed the slides under a light microscope without knowledge of patient outcome. Any cytoplasmic staining with bcl-2 was considered positive (7), indicating overexpression. Staining found in basal epithelial cells of normal prostate epithelium served as an internal control. As in a previous study (7), altered bax expression was analyzed by comparing the differences in staining intensities of both tumor and normal prostate epithelium. A higher intensity in tumor, compared with normal epithelium, indicated bax overexpression whereas a lower intensity indicated bax underexpression. Both overexpression and underexpression in different tumor regions was also seen in 2 cases; these were included in the analyses. Lymph node tissues were used as controls for bax staining.

End points and statistical analysis

Four end points were examined, including local failure (LF), distant metastasis (DM), cause-specific mortality (CSM), and overall mortality (OM), the definitions of which have previously been described in detail (15–17). All end points were measured from the date of randomization to the first reported date of failure or to the last follow-up date in the absence of failure.

The bcl-2 and bax cohorts comprised 119 and 104 patients, and there were 337 and 352 patients in the parent cohort with undetermined bcl-2 and bax data, respectively. The median follow-up of all enrolled patients was 6.7 years and the median follow-up of all living patients was 9.3 years. The distributions by bcl-2 and bax expression, patient characteristics, and treatment assignments were compared using the Chi-square test. Estimates of OM were derived by the Kaplan-Meier method (18), while estimates of LF, DM, and CSM were derived using the cumulative incidence approach (19). Multivariate analyses (MVAs) by Cox proportional hazard models (20) were applied to each of the end points to identify the impact of bcl-2 or bax.

bcl-2 was dichotomized as negative {0} vs. positive {1}. bax was categorized as same as background {0} vs. less then background {1}, greater than background {2}, or both {3}. bax was used in the MVAs as a dichotomous variable, background {0} vs. abnormal expression {1}.

RESULTS

With regard to pretreatment characteristics and assigned treatment, the bcl-2 and bax groups did not differ significantly from those in the parent cohort not assessed for the biomarkers (Table 1). Compared to the remaining unanalyzed patients, there were weak associations in the bax group with younger age ($p = 0.052$) and higher T-stage ($p = 0.08$). Tables 2 and 3 show the distribution of patients by the biomarkers and patient characteristics. bcl-2 overexpression was found in 30 (26%) cases; 13 (11%) cases were weakly positive, 8 (7%) cases were moderately positive, and 9 (8%) cases displayed strong positivity. In the bax cohort, 55 (53%) cases were classified as the same as background, 18 (17%) cases as less than background, 29

(28%) cases as greater than background, and 2 (2%) cases had cells with staining that was both weaker and stronger than background. No statistically significant relationships were seen.

In the univariate analyses shown in Table 4, neither bcl-2 nor bax were found to be significantly related to the end points tested. Similar negative results were seen in the MVAs (Tables 5 and 6). After controlling for T-stage, Gleason score, and assigned treatment, neither bcl-2 nor bax was significant. When bax was subdivided into underexpression vs. overexpression, no differences in outcomes were seen. The significant covariates for distant metastasis were Gleason score 7–10 and the absence of androgen deprivation therapy. Gleason score was also significantly related to CSM.

DISCUSSION

The bcl-2 family proteins are key regulators of apoptosis. Abnormal expression has been associated with resistance of prostatic carcinoma to radiation and androgen deprivation therapy (6,8,9,14,21). Mackey *et al.* (4) found that bcl-2 expression was higher in nonresponders to radiotherapy. When combined with data from bax expression, its pro-apoptotic heterodimerization partner, an elevated bcl-2/bax ratio was related to an increased rate of failure of radiation treatment ($p < 0.001$). Furthermore, we demonstrated previously (7) that the abnormal expression of either bcl-2 or bax in relatively favorable risk men treated with external beam radiotherapy for prostate cancer was related to an increased risk of biochemical failure ($p = 0.046$ and $p = 0.007$ for bcl-2 and bax, respectively). However, Bylund *et al.* (3) described the opposite, that bcl-2-positive patients had a longer disease-specific survival compared to bcl-2-negative patients ($p < 0.04$). The incongruity in these prior analyses prompted us to examine bcl-2 and bax expression in men treated on RTOG protocol 86–10. This is the first analysis of bcl-2 and bax expression in men treated relatively homogeneously with radiotherapy on a large multi-institutional trial.

In this report, we did not find a significant relationship between bcl-2 overexpression or altered bax expression and prostate cancer patient outcome in men treated with RT±STAD. Moreover, abnormal bcl-2 or bax expression was not significantly associated with Gleason Score or stage; although a borderline relationship of bax expression to Gleason Score was observed (Table 3). While some relationships of these biomarkers to Gleason Score have been reported (9,22–24), this has not been a consistent finding (25–27). In a report of patients with Stage B, C, or D disease, Amirghofran *et al.* (28) found that bax, but not bcl-2 expression, was significantly related to prostate carcinoma stage ($p = 0.031$); no correlation to Gleason Score was observed for either biomarker.

The patients in RTOG 86–10 examined here had more locally advanced tumors than the patients we investigated previously (7). Matsuura *et al.* (29) also found a lack of association of bcl-2 expression in patients with advanced prostate tumors (Stage C or D) to biochemical failure or disease progression after initial endocrine treatment. While bcl-2 overexpression predictably occurs in men who have progressed to distant metastasis and in those who have failed radiation or androgen deprivation therapy (6,9,12,30), pretreatment bcl-2 status in patients with locally advanced or metastatic disease may not be a dependable predictor of response.

Abnormal bax expression in our study was classified as overexpression or underexpression based on the results of a prior report (7). The initial rationale for this subdivision was that bax overexpression would be favorable in the setting of radiation due to the promotion of apoptosis, while its underexpression should lead to less of a response to radiation. However, when we separated the two states in our earlier analysis, both bax overexpression and underexpression were associated with worse outcome. Therefore, it appears that overexpression may be

representative of upstream or downstream effects that are associated with less cell killing when radiation is administered.

The results of bcl-2 and bax expression in other investigations of men with more favorable disease have been more encouraging (4,5,7). It is conceivable that early in the course of tumor growth, development of abnormal bcl-2 and bax expression better delineates patient response to treatment. There are other possible explanations for why abnormal bcl-2 and bax expression were not related to outcome. The current study was somewhat underpowered. The statistical power to detect the estimated hazard ratios ranged from 3% to 32% for the prevalence of abnormal bcl-2 and bax expression and the number of events of the endpoints tested. If the hazard ratios had been >2, the statistical power would have been 80%. However, prior studies involving similar numbers of patients have reported correlations with outcome. In addition, it is possible that antigen degradation occurred because the pretreatment archival tissue was collected more than 15 years ago. Since other biomarker associations using tissue from RTOG 86-10 have been reported (16,31,32), it is likely that most antigens have been preserved.

In conclusion, this is the first description of bcl-2 and bax expression in the diagnostic specimens of men with prostate cancer treated on a multi-institutional protocol. Neither abnormal bcl-2 nor bax was significantly associated with patient outcomes in this high-risk cohort, and there was no difference in these relationships when short-term androgen deprivation was used in conjunction with RT. A larger independent group of men with earlier-stage prostate carcinoma treated with radiotherapy should be investigated to clarify the potential role of these biomarkers.

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Table 1

Distribution of all patients by presence or absence of bcl-2 and bax data (n = 456)

Characteristics	bcl-2			bax		
	Presence (*n* = 119)	**Absence (*n* = 337)**	***p* value***	**Presence (*n* = 104)**	**Absence (*n* = 352)**	***p* value***
Age						
<75	86 (72%)	239 (71%)	0.78	82 (79%)	243 (69%)	0.052
≥75	33 (28%)	98 (29%)		22 (21%)	109 (31%)	
GLSC#						
2–6	31 (26%)	98 (32%)	0.29	27 (26%)	102 (31%)	0.33
7–10	87 (74%)	213 (68%)		76 (74%)	224 (69%)	
T-Stage						
T2	33 (28%)	104 (31%)	0.52	24 (23%)	113 (32%)	0.08
T3	86 (72%)	233 (69%)		80 (77%)	239 (68%)	
Assigned Treatment						
RT Alone	67 (56%)	163 (48%)	0.14	57 (55%)	173 (49%)	0.31
RT+STAD	52 (44%)	174 (52%)		47 (45%)	179 (51%)	

Abbreviations: GLSC = Gleason Score; RT = radiation therapy; STAD = short term androgen deprivation.

* Chi-square statistics.

Gleason score is unknown in 1 patient with bcl-2/bax data and in 26 patients with absent bcl-2/bax data.

Table 2

Distribution of patients by bcl-2 results

Characteristics	Negative (*n* = 89)	Positive (*n* = 30)	*p* value*
Age			
<75	66 (74%)	20 (67%)	0.43
≥75	23 (26%)	10 (33%)	
GLSC†			
2–6	25 (28%)	6 (21%)	0.43
7–10	64 (72%)	23 (79%)	
T-stage			
T2	24 (27%)	9 (30%)	0.75
T3	65 (73%)	21 (70%)	
Assigned treatment			
RT Alone	49 (55%)	18 (60%)	0.64
RT+STAD	40 (45%)	12 (40%)	

Abbreviations: GLSC = Gleason Score; RT = radiation therapy; STAD = short term androgen deprivation.

*Chi-square statistics.

†One patient is missing Gleason score in the “Positive” Bcl-2 group.

Table 3

Distribution of patients by bax results

Characteristics	Normal (n = 55)	Altered (n = 49)	p value*
Age			
<75	41 (75%)	41 (84%)	0.26
≥75	14 (25%)	8 (16%)	
GLSC†			
2–6	18 (33%)	9 (18%)	0.08
7–10	36 (67%)	40 (82%)	
T-stage			
T2	14 (25%)	10 (20%)	0.54
T3	41 (75%)	39 (80%)	
Assigned Treatment			
RT Alone	28 (51%)	29 (59%)	0.40
RT+STAD	27 (49%)	20 (41%)	

Abbreviations: GLSC = Gleason Score; RT = radiation therapy; STAD = short term androgen deprivation.

*Chi-square statistics.

†One patient is missing Gleason score in the “Normal” bax group.

Table 4

Univariate analysis results for bcl-2 and bax cohorts

	bcl-2		bax	
	RR (95% CI)*	***p* value†**	**RR (95% CI)***	***p* value†**
Local failure	1.12 (0.57, 2.21)	0.75	0.98 (0.51, 1.89)	0.96
Distant metastasis	1.00 (0.56, 1.79)	0.99	1.01 (0.59, 1.71)	0.98
Cause specific mortality	1.01 (0.50, 2.03)	0.98	1.30 (0.67, 2.50)	0.44
Overall mortality	0.89 (0.54, 1.46)	0.63	0.81 (0.51, 1.30)	0.39

* The relative risk (RR) and 95% confidence intervals (in parenthesis) are shown.

† *p* values were derived from the Chi-square test.

Table 5

Multivariate analysis including bcl-2 results

Endpoint	Variable[#]	Group	RR (95% CI)[*]	*p* value[†]
LF	Bcl-2	Positive	1.12 (0.57, 2.22)	0.74
	T-stage	T3	1.09 (0.53, 2.25)	0.81
	GLSC	7–10	1.39 (1.67, 2.91)	0.38
	STAD	Yes	0.63 (0.32, 1.22)	0.17
DM	Bcl-2	Positive	0.77 (0.42, 1.42)	0.40
	T-stage	T3	1.03 (0.58, 1.84)	0.93
	GLSC	7–10	2.50 (1.25, 5.02)	0.01
	STAD	Yes	0.53 (0.30, 0.92)	0.03
CSM	Bcl-2	Positive	0.87 (0.43, 1.78)	0.70
	T-stage	T3	0.98 (0.49, 1.95)	0.96
	GLSC	7–10	2.37 (1.04, 5.44)	0.04
	STAD	Yes	0.63 (0.33, 1.21)	0.17

Abbreviations: LF = local failure; DM = distant metastasis; CSM = cause specific mortality; GLSC = Gleason Score; STAD = short term androgen deprivation.

[*] Relative Risk (RR): a risk ratio of 1 indicates no difference between the two subgroups. The 95% confidence intervals are shown in parentheses.

[†] *p* value from Chi-square test using the Cox proportional hazards model.

[#] All variables were dichotomous.

Table 6

Multivariate analysis including bax results

Endpoint	Variable[#]	Group	RR (95% CI)[*]	*p*-value[†]
LF	Bax	Positive	0.96 (0.48, 1.92)	0.90
	T-Stage	T3	0.98 (0.45, 2.13)	0.95
	GLSC	7–10	1.06 (0.50, 2.23)	0.87
	STAD	Yes	0.62 (0.30, 1.24)	0.18
DM	Bax	Positive	0.83 (0.47, 1.45)	0.51
	T-Stage	T3	0.95 (0.51, 1.78)	0.87
	GLSC	7–10	2.40 (1.17, 4.93)	0.02
	STAD	Yes	0.53 (0.30, 0.96)	0.03
CSM	Bax	Positive	1.09 (0.55, 2.17)	0.80
	T-Stage	T3	0.99 (0.45, 2.16)	0.98
	GLSC	7–10	1.93 (0.82, 4.57)	0.13
	STAD	Yes	0.71 (0.36, 1.41)	0.33

Abbreviations: LF = local failure; DM = distant metastasis; CSM = cause-specific mortality; GLSC = Gleason Score; STAD = short term androgen deprivation.

[*] Relative risk (RR): a risk ratio of 1 indicates no difference between the two subgroups. The 95% confidence intervals are shown in parentheses.

[†] *p*-value from Chi-square test using the Cox proportional hazards model.

[#] All variables were dichotomous.