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Bcl-2 and Bax Expression Predict Prostate Cancer Outcome in Men Treated with Androgen Deprivation and Radiotherapy on Radiation Therapy Oncology Group Protocol 92-02

Li Yan Khor¹, Jennifer Moughan³, Tahseen Al-Saleem², Elizabeth H. Hammond⁴, Varagur Venkatesan⁵, Seth A. Rosenthal⁶, Mark A. Ritter⁷, Howard M. Sandler⁸, Gerald E. Hanks¹, William U. Shipley⁹, and Alan Pollack¹

¹ Department of Radiation Oncology, Fox Chase Cancer Center

² Department of Pathology, Fox Chase Cancer Center

³ Department of Radiation Therapy Oncology Group, American College of Radiology, Philadelphia, Pennsylvania

⁴ Department of Pathology, LDS Hospital, Intermountain Health Care, Salt Lake City, Utah

⁵ Department of Radiation Oncology, University of Western Ontario, London, Ontario, Canada

⁶ Department of Radiological Associates of Sacramento Medical Group, Inc., Sacramento, California

⁷ Department of Radiation Oncology, University of Wisconsin, Madison, Wisconsin

⁸ Department of Radiation Oncology, University of Michigan Medical Center, Ann Arbor, Michigan

⁹ Department of Radiation Oncology, Massachusetts General Hospital, Boston, Massachusetts

Abstract

Purpose—Bcl-2 is antiapoptotic, and its overexpression has been associated with resistance to androgen deprivation and poor outcome in some patients treated with radiotherapy. Bax is proapoptotic, regulating Bcl-2 through heterodimer formation. In a prior study, Bcl-2 and Bax were not related to outcome in locally advanced patients treated with radiotherapy or short-term androgen deprivation + radiotherapy (STAD+RT) on another Radiation Therapy Oncology Group trial (86-10). A follow-up investigation was carried out here in more contemporary high-risk men treated on Radiation Therapy Oncology Group 92-02 with STAD+RT or long-term AD+RT (LTAD+RT).

Experimental Design—Adequate tissue was available to be analyzed immunohistochemically in 502 patients for Bcl-2 and 343 patients for Bax. Univariate and multivariate analyses by Cox proportional hazards models were applied to end points of failure.

Results—Bcl-2 was positive in 45.6% cases, and Bax expression altered in 53.9% cases. Abnormal Bcl-2 was not related to any of the failure end points tested. Altered Bax expression was significantly associated with any failure ($P = 0.023$) and marginally with biochemical failure ($P = 0.085$). The combination of negative Bcl-2/normal Bax expression seemed more robust, being significantly related to reduced biochemical failure ($P = 0.036$) and any failure ($P = 0.046$). The predictive value

Requests for reprints: Alan Pollack, Department of Radiation Oncology, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111 Phone: 215-728-2940; Fax: 215-728-2868; Alan.Pollack@FCCC.EDU.

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of negative Bcl-2/normal Bax was most pronounced in those who received STAD+RT, as opposed to LTAD+RT

Conclusions—Normal Bax expression was associated with significantly more favorable outcome. The combination of negative Bcl-2 and normal Bax was more consistently significant, particularly when STAD+RT was the treatment administered. These data suggest that LTAD+RT should be used when either Bcl-2 or Bax is abnormally expressed.

The Bcl-2 family proteins consist of members with opposing functions, interacting with one another to achieve a balance between cell survival and apoptosis signals. Bcl-2, a pro-survival member found in the basal epithelium of the prostate, has been extensively studied in prostate cancer. Androgen deprivation treatment causes an increase in Bcl-2 expression (1–4), and the overexpression of Bcl-2 is associated with the development of androgen independence (1,5, 6). Our *in vitro* studies also show Bcl-2 suppression by antisense Bcl-2 sensitizes prostate cancer cells to radiation (7).

Conversely, Bax is proapoptotic (8,9). Bax expression is primarily in the secretory epithelial cells of the prostate and thus more susceptible to apoptosis caused by androgen deprivation (10). *In vivo* studies show that Bax up-regulation temporarily induces apoptosis in the setting of androgen deprivation (11). Indeed, it has even been shown that in androgen-independent tumors that are androgen receptor positive, reintroduction of androgen reduces tumor growth via Bax-mediated apoptosis (12). The influence of these proteins on persistent localized prostate cancer characterizes them as highly suitable candidates for prognostic markers of the disease.

Previous analyses of Bcl-2 and Bax expression by our group and others involved men treated with definitive radiotherapy alone (13,14) and short-term androgen deprivation + radiotherapy (STAD+RT; ref. 14). In a study including patients with a range of risk factors (e.g., T₁-T₃), abnormal expression of both proteins was associated with treatment failure (13). In another cohort of more locally advanced patients [Radiation Therapy Oncology Group (RTOG) trial 86-10], neither Bcl-2 nor Bax was predictive of outcome (14); prostate-specific antigen (PSA) data were not available in this older cohort. Both studies were also limited in sample size.

The present study investigates the value of Bcl-2 and Bax expression in a more contemporary high-risk group of men who were enrolled in the phase III randomized trial (RTOG 92-02). RTOG 92-02 builds on the prior RTOG experience, comparing STAD+RT with long-term AD +RT (LTAD+RT).

Materials and Methods

Study population

RTOG protocol 92-02 has been previously described (15,16). There were 1,518 assessable patients. Of these, 502 pretreatment diagnostic needle-core biopsies or transurethral resection tumor specimens were acquired and adequately stained for Bcl-2 (and 343 for Bax) expression. Sixty additional cases stained for Bax showed high staining levels with excessive background and were unsuitable for analysis. The slides stained for Bax were also those cut closer to the end of the blocks, and 99 cases were found to be lacking in tissue or adequate tumor.

The median age of the Bcl-2 study cohort was 70 years (range, 43–88 years), median initial pretreatment PSA (iPSA) was 20.7 ng/mL (range, 0.9–219.7 ng/mL), and 283 (56.4%) patients had T₃-T₄ disease. Median follow-up for patients alive in the Bcl-2 cohort was 10.4 years. The Bax study group had a median age of 71 years (range, 49–86 years) and median iPSA of 21.7 ng/mL (range, 0.8–151.1 ng/mL), and 179 (52.2%) patients had T₃-T₄ disease. Median follow-up for patients alive in the Bax cohort was 10.5 years.

Immunohistochemical technique

The immunohistochemical protocol was detailed previously (14). The primary antibodies used were Bcl-2 (clone 124, DAKO Corp.; 1:100 dilution) and Bax (clone 2D2, Zymed Laboratories, Inc.; 1:200 dilution). The labeled streptavidin-biotin (LSAB) immunohistochemical method was used (DAKO LSAB 2 kit) followed by a diaminobenzidine chromagen and a hematoxylin counterstain (DAKO) for visualization. All staining was done on an autostainer (DakoCytomation).

The investigators had no knowledge of patient outcome. The first investigator (L-Y.K.) independently examined the slides for cytoplasmic staining intensity under light microscopy. A tissue microarray control consisting of four plugs of prostate cancer tissue, each expressing a different intensity (0–3), was referenced to rate the intensity of each case. A cluster of >20 positive tumor cells was considered Bcl-2 overexpression. When two or more intensities were encountered, the highest intensity was recorded. Normal prostate epithelium also served as an internal control for Bcl-2 staining. Underexpression or over-expression of Bax relative to the staining intensities of neighboring normal prostate epithelial cells were considered “altered Bax expression” (13,14). Both the prostate cancer tissue microarray control and a separate human lymph node tissue were used as Bax staining controls. All the cases were then reviewed with an oncologic surgical pathologist with a special interest in urological pathology (T.A-S.). Both investigators sat at a multi-headed microscope and discussed each case. Minor variations in the grading of staining intensity between the investigators was seen in 130 Bcl-2 and 170 Bax cases. These differences would not have altered the final classification of Bcl-2–positive (any staining) or altered Bax staining.

Definition of end points

Two definitions were considered for biochemical failure: the American Society of Therapeutic Radiology and Oncology (ASTRO) consensus definition (ASTRO BF), three consecutive increases in PSA or a posttreatment PSA nadir > 4.0 ng/mL (17) and the Phoenix definition (BF Phoenix), PSA > nadir + 2 ng/mL after treatment (18). Local failure was assessed by palpation and defined as tumor growth of 25% or local persistent disease beyond 18 months. Distant metastasis was defined as clinical evidence of distant disease by any method. The failure events for any failure were defined as all occurrences (local failure, regional nodal failure, distant failure, or ASTRO BF) except death. Cause-specific mortality was death certified as due to prostate cancer, death due to treatment complications, death from unknown causes with active malignancy (clinical disease relapse), or death from another cancer with documented bone metastases attributed to prostate cancer before the appearance of the second independent cancer. Finally, overall mortality was defined as death due to any cause.

Statistical analysis

All pretreatment characteristics were dichotomized. Age was dichotomized by the median age in the entire cohort (<70 versus ≥70 years), iPSA and T stage by their stratification groupings in RTOG 92-02 (≤30 versus >30 ng/mL; T_{2b}/T_{2c} versus T₃/T₄), and Gleason score as 2 to 6 versus 7 to 10. The χ^2 test and Cox proportional hazards model (19) were applied to assess if the missing Bcl-2 and Bax data were dependent on pretreatment characteristics, treatment arm, or outcome. Actuarial estimates for overall mortality were based on Kaplan-Meier methods (20), and the cumulative incidence method was used to estimate cause-specific mortality, distant metastasis, local failure, biochemical failure, and any failure (21).

Results

Bcl-2 was overexpressed in 229 (45.6%) of the cases studied. Altered Bax was seen in 185 (53.9%) cases: 26 (7.6%) cases had Bax underexpression, and 159 (46.4%) cases had Bax over-

expression. In prior studies, either type of abnormal Bax expression was associated with an adverse outcome (13,14); thus, they were considered together here.

Comparisons were made between cases that were assessed for Bcl-2 ($n = 502$) and those that were not ($n = 1,016$). There were no significant differences in age, iPSA, or T stage. Those with Bcl-2 data had a greater proportion of Gleason score 7 to 10 (65.5% versus 55.9%; $P = 0.0005$), and more cases were assigned to the LTAD+RT arm (53.6% versus 47.9%; $P = 0.0382$). Likewise for Bax, the distributions of patients by pretreatment characteristics were similar, except a greater proportion in the Bax cohort had Gleason score 7 to 10 (64% versus 57.7%; $P = 0.0423$), and more cases were assigned to LTAD+RT (55.4% versus 48.2%; $P = 0.0186$) compared with the cases in which Bax staining was not available. There were no statistical differences between the biomarker (Bcl-2 or Bax) available and unavailable groups in terms of outcome by all the end points (overall mortality, cause-specific mortality, distant metastasis, local failure, biochemical failure, or any failure) tested.

Table 1 shows the distribution of patients by Bcl-2 and Bax staining, pretreatment prognostic factors, and assigned treatment. Bcl-2 overexpression was significantly related to Gleason score 7 to 10 disease (70.7% versus 61.2%; $P = 0.0305$), whereas altered Bax expression and the combined results were not related to any pretreatment factor (age, Gleason score, iPSA, and T stage) or assigned treatment.

In the univariate analyses, Bcl-2 positivity was significantly related to overall mortality [relative risk (RR), 1.31; 95% confidence interval (95% CI), 1.03–1.67; $P = 0.0307$] and distant metastasis (RR, 1.51; 95% CI, 1.02–2.23; $P = 0.0387$). However, in multivariate analyses, Bcl-2 overexpression was not independently related to these or any of the other end points tested, although the RRs for failure were higher for overall mortality, cause-specific mortality, distant metastasis, and local failure (Table 2). In univariate analyses, altered Bax expression was not significantly associated with any of the end points tested, although there was a trend for any failure (RR, 1.30; 95% CI, 0.97–1.73; $P = 0.0806$). In the multivariate analyses (Table 3), abnormal Bax expression was significantly associated with any failure (RR, 1.43; 95% CI, 1.05–1.95; $P = 0.0226$) and marginally with biochemical failure using the ASTRO definition (RR, 1.37; 95% CI, 0.96–1.97; $P = 0.0851$).

Because both Bcl-2 and Bax seem to contribute to the apoptotic response of prostate tumor cells to radiation and androgen deprivation (22–24), the two biomarkers were examined together. We first divided the patients into three groups: negative Bcl-2 and normal Bax expression (negative Bcl-2/normal Bax) versus either positive Bcl-2 or abnormal Bax expression versus positive Bcl-2/abnormal Bax expression. The first group was segregated from the other two. The results displayed summarize the comparisons of negative Bcl-2/normal Bax versus the other possible combinations of positive Bcl-2 and/or altered Bax expression. In univariate analyses, negative Bcl-2/normal Bax was significantly related to both any failure (RR, 1.44; 95% CI, 1.01–2.03; $P = 0.0408$) and ASTRO BF (RR, 1.62; 95% CI, 1.06–2.48; $P = 0.0242$). In the multivariate analyses (Table 4), the relationships remained significant for any failure (RR, 1.45; 95% CI, 1.01–2.10; $P = 0.0457$) and biochemical failure (RR, 1.60; 95% CI, 1.03–2.49; $P = 0.0359$). The failure curves for these end points are displayed in Fig. 1. These results seemed more robust than the relationships seen with Bax alone.

Finally, the relationship of Bcl-2/Bax expression to length of androgen deprivation therapy (assigned treated in the protocol) was tested. Table 5 shows that the 5-year failure rates (biochemical failure and any failure) were much greater for patients who had positive Bcl-2 and/or altered Bax tumors when STAD+RT was used. Although there was a trend for worse outcome in patients with negative Bcl-2/normal Bax tumors after STAD+RT, compared with LTAD+RT, the association was much less apparent. In the setting of LTAD+RT, positive Bcl-2

and/or altered Bax expression was not related to increased failure; the failure rates were about the same as for patients with negative Bcl-2/normal Bax expression.

Discussion

The predictive value of Bcl-2 and Bax in relation to clinical outcome has been investigated in various correlative studies on prostate cancer, and the results have been contradictory or inconclusive (13,14,22,24–27). The reasons for this may include variations in clinical characteristics of the study cohorts, different scoring methods, and low statistical power. The overwhelming evidence for the involvement of Bcl-2 and Bax in prostate cancer recurrence (22,24,27) and progression to androgen independence (1,5,6,28–30) highlight the need for further studies of these biomarkers. The current investigation is, to our knowledge, the largest multi-institutional study of Bcl-2 and Bax expression in contemporary high-risk prostate cancer patients.

The study population described herein was derived from patients treated on RTOG protocol 92-02 with STAD+RT or LTAD+RT. There were some slight differences in patient characteristics between those in the study cohort (Bcl-2 and/or Bax available) and the remaining biomarker unavailable patients. However, there were no differences in patient outcome between the two groups using a number of end points.

The approach used was modeled after that described in two prior reports from our group (13, 14). We initially reported that both Bcl-2 overexpression (16% of cases) and abnormal (underexpression or overexpression) Bax expression (23% of cases) were significant predictors of biochemical failure in men treated with external beam radiotherapy alone at a single institution in the PSA era. The associations of Bcl-2 and Bax expression were independent of iPSA, Gleason score, T stage, and Ki-67 staining. The best outcome was seen in those whose tumors had a negative Bcl-2/normal Bax pattern.

In the second study, Bcl-2 overexpression (26% of cases) and altered Bax expression (47% of cases) were measured in pretreatment diagnostic tissue samples from RTOG 86-10, a randomized trial comparing radiotherapy alone with STAD+RT. The STAD+RT treatment in this protocol was identical to the STAD+RT treatment in the current analysis of patients in RTOG 92-02. Neither Bcl-2 nor Bax was predictive of patient outcome. We did not combine Bcl-2 and Bax expression in the analysis of 86-10 patients because there was no suggestion of a relationship with either marker when tested individually. The patients in this trial were locally advanced and were treated in the pre-PSA era. The patients in RTOG 92-02 represent more contemporary high-risk patients.

In both prior studies of Bcl-2 and Bax expression from our group, the number of cases examined was in the 100 to 120 range. Biomarker studies are typically underpowered. An important feature of the analysis described here is that it involves much greater numbers and, consequently, power to show significance. Bcl-2 overexpression was seen in 46% and abnormal Bax expression in 54%. These percentages are higher than in our prior studies, possibly reflecting better preservation of antigens. It should be noted that the levels of Bcl-2 overexpression and abnormal Bax expression in samples from RTOG 92-02 are the same as for prospectively analyzed samples in another single institution cohort of men treated with external beam radiotherapy (31).

In multivariate analyses of cases from RTOG 92-02, Bcl-2 overexpression was not associated with any outcome measure, whereas altered Bax expression was significantly associated with any failure and was borderline significant for ASTRO BF. The most consistent relationship was seen when Bcl-2 and Bax expression were considered together. These data are concordant with our first analysis (13) and other reports of combining Bcl-2 and Bax (22). Some have

advocated the Bcl-2/Bax ratio as being important (32–35), but in our hands, Bax overexpression has been a determinant of adverse outcome, and the Bcl-2/Bax ratio is not applicable.

We also investigated the influence of Bcl-2 and Bax expression on patient outcome when stratified by length of androgen deprivation. There was an apparent benefit in giving LTAD +RT to patients with positive Bcl-2 and/or abnormal Bax expression, with similar more favorable results to those who had negative Bcl-2/normal Bax (Table 5). The greatest risk of failure was seen in men who received STAD+RT and who had positive Bcl-2 and/or abnormal Bax.

In conclusion, Bcl-2 and Bax expression were most predictive of outcome when combined, although at this point, the associations seen were limited to ASTRO BF and any failure. The data suggest that men with tumors overexpressing Bcl-2 and/or altered Bax expression should receive LTAD+RT as opposed to STAD+RT. The RTOG translational research group has been investigating a series of potential biomarkers in tissue from patients in protocol 92-02 including Ki-67, p53, MDM2, p16 (16,36,37), and now Bcl-2 and Bax. The aim is to ultimately establish a consensus biomarker panel for risk stratification of high-risk prostate cancer patients. The data described suggest that Bcl-2 and Bax should be included, after which the panel will be validated on an independent patient cohort.

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Fig. 1.

A, failure curve of ASTRO BF by combined Bcl-2/Bax expression. *B*, failure curve of any failure by combined Bcl-2/Bax expression.

Table 1
Distribution of patients by Bcl-2 (n = 502) and Bax results (n = 343)

Characteristics	Bcl-2			Bax*		
	Negative, n (%)	Positive, n (%)	P†	Normal, n (%)	Altered, n (%)	P‡
Eligible	273 (54.4)	229 (45.6)	—	158 (46.1)	185 (53.9)	—
Age (y)						
<70	129 (47.3)	100 (43.7)	0.4219	71 (44.9)	73 (39.5)	0.3056
≥70	144 (52.7)	129 (56.3)		87 (55.1)	112 (60.5)	
Gleason score§						
2–6	99 (38.8)	63 (29.3)	0.0305	55 (37.9)	59 (34.3)	0.5024
7–10	156 (61.2)	152 (70.7)		90 (62.1)	113 (65.7)	
iPSA (ng/mL)						
≤30	182 (66.7)	160 (69.9)	0.4431	101 (63.9)	124 (67.0)	0.5465
>30	91 (33.3)	69 (30.1)		57 (36.1)	61 (33.0)	
Clinical stage						
T ₂	123 (45.1)	96 (41.9)	0.4807	76 (48.1)	88 (47.6)	0.9214
T ₃ or T ₄	150 (54.9)	133 (58.1)		82 (51.9)	97 (52.4)	
Assigned treatment						
STAD+RT	123 (45.1)	110 (48.1)	0.5049	72 (45.6)	81 (43.8)	0.7402
LTAD+RT	150 (54.9)	119 (52.0)		86 (54.4)	104 (56.2)	

* Seventeen patients with negative (no stain) Bax were excluded.
† P values from χ^2 test.
‡ Eighteen and 14 cases with negative and positive Bcl-2 data, respectively, and 13 cases each with normal and altered Bax data have unknown Gleason scores.

Table 2Multivariate analysis of Bcl-2 staining ($n = 470$)

End point [*]	Covariate	Group	HR [†] (95% CI)	P [‡]
OM	Bcl-2 intensity	Positive	1.24 (0.96–1.60)	0.1015
CSM	Bcl-2 intensity	Positive	1.24 (0.79–1.94)	0.3450
DM	Bcl-2 intensity	Positive	1.37 (0.90–2.08)	0.1479
LF	Bcl-2 intensity	Positive	1.27 (0.78–2.07)	0.3409
BF Phoenix [§]	Bcl-2 intensity	Positive	1.12 (0.87–1.45)	0.3744
All covariates displayed for ASTRO BF and AF				
BF ASTRO [§]	Bcl-2 intensity	Positive	1.13 (0.85–1.51)	0.3863
	Assigned Tx	STAD+RT	3.01 (2.24–4.04)	<0.0001
	iPSA	>30	1.53 (1.14–2.05)	0.0043
	Gleason score ^{//}	7–10	1.09 (0.80–1.48)	0.5872
	T stage	T ₃ /T ₄	1.03 (0.77–1.37)	0.8444
AF	Bcl-2 intensity	Positive	1.16 (0.91–1.48)	0.2245
	Assigned Tx	STAD+RT	2.19 (1.72–2.79)	<0.0001
	iPSA	>30	1.30 (1.01–1.68)	0.0440
	Gleason score	7–10	0.97 (0.75–1.25)	0.7957
	T stage	T ₃ /T ₄	1.01 (0.79–1.29)	0.9385

Abbreviations: HR, hazard ratio; OM, overall mortality; CSM, cause-specific mortality; DM, distant metastasis; LF, local failure; BF, biochemical failure; Tx, treatment; AF, any failure.

* In the top half of the table, only the results of Bcl-2 staining are shown, although for each end point tested, all of the covariates displayed in the bottom half of the table were used.

[†] Hazard ratios were calculated using the multivariate Cox model.

[‡] P values from χ^2 test using the Cox proportional hazards model.

[§] Nine cases have unknown BF status ($n = 461$).

^{//} Thirty-two cases have unknown Gleason scores.

Table 3Multivariate analysis of Bax intensity ($n = 317$)

End point [*]	Covariate	Group	HR [†] (95% CI)	P [‡]
OM	Bax intensity	Altered	0.99 (0.73–1.34)	0.9371
CSM	Bax intensity	Altered	1.05 (0.60–1.84)	0.8560
DM	Bax intensity	Altered	1.06 (0.63–1.80)	0.8139
LF	Bax intensity	Altered	0.95 (0.54–1.67)	0.8517
BF Phoenix [§]	Bax intensity	Altered	1.15 (0.84–1.58)	0.3942
All covariates displayed for ASTRO BF and AF				
BF ASTRO [§]	Bax intensity	Altered	1.37 (0.96–1.97)	0.0851
	Assigned Tx	STAD+RT	3.28 (2.26–4.76)	<0.0001
	iPSA	>30	1.64 (1.14–2.36)	0.0074
	Gleason score	7–10	1.21 (0.82–1.79)	0.3336
	T stage	T ₃ /T ₄	1.29 (0.89–1.85)	0.1745
AF	Bax intensity	Altered	1.43 (1.05–1.95)	0.0226
	Assigned Tx	STAD+RT	2.33 (1.71–3.16)	<0.0001
	iPSA	>30	1.30 (0.95–1.78)	0.1033
	Gleason score	7–10	0.96 (0.70–1.33)	0.8265
	T stage	T ₃ /T ₄	1.18 (0.87–1.61)	0.2794

NOTE: Twenty-six cases had unknown Gleason scores, and 17 patients were unassessable for Bax (internal control negative).

* In the top half of the table, only the results of Bax staining are shown, although for each end point tested, all of the covariates displayed in the bottom half of the table were used.

[†] All hazard ratios were calculated using the multivariate cox model.

[‡] P values from χ^2 test using the Cox proportional hazards model.

[§] Seven cases had unknown BF status ($n = 310$).

Table 4
Multivariate analysis of combined Bcl-2/Bax intensities

End point	Covariate	Group	HR* (95% CI)	P [†]
BF ASTRO [‡]	Bcl-2/Bax	Positive and/or altered	1.60 (1.03–2.49)	0.0359
	Assigned Tx	STAD+RT	3.14 (2.16–4.56)	<0.0001
	iPSA	>30	1.71 (1.19–2.47)	0.0041
	Gleason score	7–10	1.13 (0.76–1.67)	0.5547
	T stage	T ₃ /T ₄	1.23 (0.85–1.77)	0.2763
AF [§]	Bcl-2/Bax	Positive and/or altered	1.45 (1.01–2.10)	0.0457
	Assigned Tx	STAD+RT	2.26 (1.66–3.08)	<0.0001
	iPSA	>30	1.34 (0.97–1.84)	0.0741
	Gleason score	7–10	0.91 (0.66–1.26)	0.5591
	T stage	T ₃ /T ₄	1.16 (0.85–1.59)	0.3367

* All hazard ratios were calculated using the multivariate Cox model.

[†] P values from χ^2 test using the Cox proportional hazards model.

[‡] Restricted to patients with Bcl-2/Bax, Gleason score, and BF data ($n = 295$).

[§] Restricted to patients with Bcl-2/Bax, Gleason score, and BF data ($n = 302$).

Table 5
Five-year rates by length of treatment using combined Bcl-2/Bax intensities (*n* = 326)

End point	Group	Assigned treatment	<i>n</i>	Failure rate* (%)	<i>P</i> [†]
BF ASTRO [‡]	Negative Bcl-2/normal Bax	STAD+RT	36	33.3	0.116
		LTAD+RT	51	21.6	
	Positive Bcl-2 and/or altered Bax	STAD+RT	106	61.3	<0.0001
		LTAD+RT	126	23.9	
AF [‡]	Negative Bcl-2/normal Bax	STAD+RT	27	48.7	0.056
		LTAD+RT	51	31.4	
	Positive Bcl-2 and/or altered Bax	STAD+RT	107	69.2	<0.0001
		LTAD+RT	131	38.2	

*The cumulative incidence method was used to estimate the 5-y failure rates.
[†]*P* values from the Gray's test.
[‡]One case from the negative and normal group and six cases from the positive and/or altered group had unknown BF status.