Bcl-2 Expression as a Predictive Marker of Hormone-Refractory Prostate Cancer Treated with Taxane-Based Chemotherapy

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Abstract

Purpose: Bcl-2 inhibits apoptosis, and its overexpression is associated with hormone refractory prostate cancer (HRPC). Bak and Bax are in the Bcl-2 family and counteract the antiapoptotic function of Bcl-2. Taxane-induced (paclitaxel and its analogue docetaxel) phosphorylation of Bcl-2 abolishes the potential antiapoptotic effect of Bcl-2. We hypothesized that (*a*) survival benefit in HRPC patients treated with taxanes is determined by the presence of Bcl-2 protein and (*b*) altered expression of Bak and Bax protein caused by genetic mutation is associated with biological aggressiveness of prostate cancer.

Experimental Design: Forty localized prostate cancer and 30 HRPC cases were used in this study. Surgical specimens of localized prostate cancer and biopsy specimens of HRPC were used for immunostaining of Bcl-2, Bak, and Bax as well as DNA extraction. Mutations in the *Bak* and *Bax* genes were screened by single-strand conformational polymorphism, and confirmed by direct DNA sequencing.

Results: Bcl-2–positive HRPC showed longer cause-specific survival in comparison with the counterparts. Multivariate analysis revealed that the level of Bcl-2 expression before treatment with taxane-based chemotherapy was an independent predictor for cause-specific survival ($P < 0.01$) and baseline prostate-specific antigen level was an independent predictor for progression-free survival ($P < 0.01$). *Bax* gene mutation was found in only one HRPC specimen.

Conclusions: Bcl-2 expression in addition to prostate-specific antigen measurement before treatment could identify HRPC patients who may benefit from taxane-based chemotherapy. Mutation of the *Bak* and *Bax* genes is a rare event in prostate cancer.

Abnormal balance between antiapoptotic and proapoptotic molecules may affect tumor development and/or progression. The Bcl-2 family plays a central role in the regulation of apoptosis, which is induced by a wide variety of stimuli (1). Members of this protein family can be divided into death antagonists, such as Bcl-2, and death agonists, such as Bak (Bcl-2 homologous antagonist/killer; refs. 2, 3) and Bax (Bcl-2 associated X; ref. 4). The different members of the Bcl-2 family of proteins can form homodimers or heterodimers with other members of the same family through highly conserved amino acid residues of the homologous domains, BH-1, BH-2, and BH-3 (2–5). The ratio of death agonists to antagonists determines the susceptibility to death stimuli (6). Bcl-2 is an inner mitochondrial membrane protein that inhibits apoptosis, conferring a survival advantage on cells expressing this oncoprotein (7). In normal and hyperplastic prostatic tissues, Bcl-2 protein is expressed in the cytoplasm of basal epithelial cells (8). The prevalence of Bcl-2 overexpression is lower in localized prostate cancer compared with hormone refractory prostate cancer (HRPC; refs. 8–11). Overexpression of Bcl-2 seems to enable the prostate cancer cells to survive in an androgen-deprived environment, and to confer resistance to antiandrogen therapy (9). The functional role of Bak and Bax, which are in the same protein family as Bcl-2 and were cloned as Bcl-2–related genes, is to inhibit the protection from apoptosis that is provided by Bcl-2 (12, 13). Genetic alteration of the *Bak* or *Bax* gene has been reported in hematopoietic cancer (14) and colorectal cancer (15, 16), suggesting that mutational changes of proapoptotic genes might contribute to tumor development and/or progression in some subsets of cancers. To our knowledge, such mutational analyses are limited in prostate cancer.

The vast majority of metastatic prostate cancer patients initially respond to androgen ablation; however, most will eventually develop hormone refractory disease. Unfortunately, no definite treatment modalities have been available for HRPC until these days. However, we have reported that taxane-based chemotherapy was effective against some cases of HRPC (17, 18). In addition, most recently, two pivotal multicenter phase III randomized clinical trials, TAX 327 and Southwest Oncology Group 9916, have shown a survival advantage of HRPC treated with taxane-based chemotherapy (19, 20). On the basis of these findings, taxane-based chemotherapy is now

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considered standard first-line therapy for HRPC in the United States.

Studies using inhibitors of phosphatases have shown that Bcl-2 can be phosphorylated at serine residues and that Bcl-2 phosphorylation is associated with its loss of function (21).Treatment of PC-3 cells with taxanes, which affect microtubule integrity, has been shown to induce Bcl-2 phosphorylation and apoptosis at G_2-M. However, treatment of Bcl-2 – negative DU-145 prostate cancer cells with taxanes did not induce apoptosis (22, 23). Thus, Bcl-2 might be a very useful target for chemotherapy in HRPC. Considering that cancer cells that overexpress Bcl-2 are sensitive to taxane-induced apoptosis, therapeutic response and survival benefit of HRPC patients treated with taxane-based chemotherapy may depend on the presence of Bcl-2 protein. In the present study, we examined the expression of Bcl-2, Bak, and Bax protein in human prostate cancer tissues, focusing on the relationship between expression of Bcl-2 and therapeutic response and survival benefit of taxane-based chemotherapy in HRPC patients.

Materials and Methods

Patients. A total of 79 cases were enrolled in this study. Forty patients (median age 72.0 years, range 56-77 years) underwent radical prostatectomy for localized prostate cancer at Shimane University Hospital between April 2000 and April 2001. All 40 cases were primary and untreated prostate cancer at the initial presentation. Each prostate cancer specimen was clinically classified according to the Unio Internationale Contra Cancrum staging system (24). The preoperative staging strategy included prostate-specific antigen (PSA) measurement, transrectal ultrasonography, computed tomography, chest X-rays, bone scans, and magnetic resonance imaging. The remaining 39 cases (median age 75.0 years, range 54-80 years) were those diagnosed with HRPC who underwent prostatic biopsy at our institution consecutively between June 1997 and December 2001. Of the 39 cases, nine were excluded from this study, as their biopsy specimens at the time of HRPC diagnosis did not show any viable cancer cells. Thus, 30 cases (median age 72.5 years, range 62-80 years) were recruited from these 39 HRPC cases and treated with 100 mg/m^2 paclitaxel or 30 mg/m^2 docetaxel i.v. weekly, 10 mg/m^2 estramustine phosphate orally daily, and carboplatin i.v. to an area under the curve of 6 on day 1 of every 4-week cycle (17, 18). In addition, prostatic biopsy specimens of another consecutive 19 HRPC cases (median age 78.0 years, range 67-80 years) who were not exposed to taxane-based chemotherapy as proper controls for HRPC treated with taxane-based chemotherapy were also examined for Bcl-2, Bak, and Bax expression at the time of HRPC diagnosis between January 1995 and December 2001; all of these specimens showed viable cancer cells.

Evaluations of therapeutic response in HRPC patients. Pretreatment evaluation included PSA measurement, transrectal ultrasonography, computed tomography, chest X-rays, bone scans, and magnetic resonance imaging. The HRPC cases were evaluated for response by imaging studies and systematic sextant biopsies of the prostate every two cycles. In patients with measurable disease, standard phase II response criteria were used (25). A complete response required complete disappearance of all disease, and partial response required a ≥50% reduction in the sum of the products of the perpendicular diameters of all lesions. Stable disease required a <50% reduction or a ≤25% increase in the sum of the products of the perpendicular diameters of all lesions. Changes in intensity or size of osseous lesions using bone scans were difficult to interpret. Therefore, the appearance of one or more new osseous lesions was required on bone scan to identify progressive disease. Likewise, disappearance of one or more and all lesions was required to identify partial response and complete response, respectively. No change in the number of osseous lesions was required to identify stable disease. PSA level at baseline had to be ≥4 ng/mL to assess posttherapy changes. The posttherapy change in PSA level was defined by the degree of change from baseline. Progression of PSA level was defined as three consecutive increases in PSA of 50% over the nadir value at a minimum of 4 ng/mL, at least 4 weeks apart. Systematic sextant biopsies were evaluated for antitumor therapeutic effect (e.g., degeneration and disappearance of viable cells) and reduction in the number of positive biopsy specimens, and were classified as improvement, no change, or progression by a pathologist. Combined response criteria used in this study were defined based on the Response Evaluation Criteria in Solid Tumors and PSA Working Group criteria (26, 27). Briefly, complete response required complete disappearance of all evidence of all at sites of measurable or osseous disease with normalization of PSA (defined as PSA ≤4 ng/mL). Moreover, disappearance of viable cells by systematic sextant biopsy was compulsory for determination of complete response. Partial response was defined as a ≥50% reduction in the sum of the products of the perpendicular diameters of all measurable lesions, and a ≥50% reduction in PSA without the appearance of new osseous lesions or worsening of pathologic findings. Progressive disease was defined as progression of PSA levels, measurable or osseous lesions, or worsened pathologic findings. In addition, these response criteria have been used in our recent reports associated with HRPC (17, 18). Time to progression was measured from the first day of treatment to the off-study data or progressive disease. Cause-specific survival was measured from the initiation of therapy to the date of death or last follow-up. In the 19 control cases of HRPC, both were calculated from the day of HRPC diagnosis.

Tissue preparation. Surgical specimens of localized prostate cancer and biopsy specimens of HRPC were fixed in 10% buffered formalin (pH 7.0) for 12 hours and embedded in paraffin wax. Five consecutive sections of 5 μm thickness were cut from each block and used for H&E staining for histologic evaluation.

DNA extraction. Localized prostate cancer genomic DNA from cancerous and matched normal prostate areas was extracted using a DNA mini kit (Qiagen, Valencia, CA). From paraffin-embedded biopsy specimens of HRPC, genomic DNA was microscopically dissected and extracted using DEXPAT (TaKaRa, Kyoto, Japan).

Immunostaining. Immunostaining of Bcl-2, Bak, and Bax was done on 5-μm-thick consecutive sections obtained from paraffin-embedded materials, using mouse monoclonal antibody for Bcl-2 (1:25 dilution; clone 124, DAKO, Glostrup, Denmark), rabbit polyclonal antibody for Bak (1:50 dilution; DAKO, Carpinteria, CA), and rabbit polyclonal antibody for Bax (1:25 dilution; DAKO, Carpinteria, CA). Slides were prepared for antigen retrieval using citrate buffer [10 mmol/L (pH 6.0)] before incubation of the primary antibody. Bcl-2 immunoreactivity was detected using a LSAB kit (DAKO, Kyoto, Japan), whereas Bak and Bax immunostaining was done using an Envision system (DAKO, Carpinteria, CA). The chromogen 3,3′-diaminobenzidine (Sigma-Aldrich, St. Louis, MO) was used and sections were counterstained with hematoxylin. For the negative control, the primary antibody was replaced with a nonimmune serum. Normal human tonsilla and urothelium (urinary bladder) served as positive controls of Bcl-2, Bax, and Bak, respectively.

Evaluation of immunostaining. All slides were independently reviewed by two observers (T.Y. and M.Y.). Both observers were qualified and blinded to all clinical outcome data. When a discrepancy was noted between the two results, the observers discussed the slides and a common result was reached. At least 2,000 tumor cells in 20 randomly selected high power fields (×400) for localized prostate cancer and at least 400 tumor cells encountered in five randomly selected high power fields (×400) for HRPC were analyzed. We regarded tumor cells whose intensity was similar to that of the positive control as positively stained. The positive rate was expressed as the mean percentage of positively stained tumor cells against the total number of tumor cells counted. A specimen was regarded as positive when >10% of the tumor cells showed immunoreactivity. To minimize the risk of false

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positives, a cutoff value of 10% positive cells was used to define Bcl-2, Bak, and Bax positivity for a tumor. Because of the lack of generally accepted standards for quantitation of Bcl-2, Bak, and Bax immunostaining, the selection of this cutoff was based on previous reports from the literature (28–31). Additionally, because biopsy specimens of borderline HRPC cases have revealed strong-intensity immunohistochemical staining and statistical study has not been done, intensity was not used as a factor in immunoreactivity assessment in this study.

Single-strand conformational polymorphism and DNA sequencing. Primers within the intronic sequences were used to amplify the entire coding sequence of the *Bak* and *Bax* genes in five and six fragments, respectively. All primers encompassed the regions of the intron/exon boundaries. Primer sequences are shown in Table 1. A genomic DNA sample (100 ng) was diluted into 20 μL solution containing 20 mmol/L deoxynucleotide triphosphates, 500 nmol/L of each primer, 0.5 units KOD DNA polymerase, and PCR reaction buffer provided by the manufacturer (TOYOBO, Osaka, Japan). DNA samples were amplified through 45 cycles on a Thermal Cycler (MJ Research, Watertown, MA) at 94°C for 30 seconds of denaturation, 58°C for 30 seconds of annealing, and 68°C for 45 seconds of extension. In each round of PCR, a tube without template DNA served as a negative control. Single-strand conformational polymorphism (SSCP) analysis was done with a nonradioactive approach using a 12% polyacrylamide gel (32). Electrophoresis was done using Tris-glycine buffer cooling at 10°C or 20°C for 5 hours at 55 W constant power. In the case of abnormal band shifts, purified PCR product was used as the template for direct sequencing. Direct sequencing by ABI PRISM BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA) using the same primers as PCR amplification was done in both directions according to the instructions of the manufacturer.

Statistical analysis. All numerical data are expressed as mean ± SD. Relationships between expression of Bcl-2, Bak, or Bax protein and clinicopathologic findings in localized prostate cancer were analyzed using the Kruskal-Wallis test. The difference in immunostaining between localized prostate cancer samples and HRPC samples was analyzed by the Mann-Whitney test. The relationship between immunostaining and therapeutic response to chemotherapy in HRPC was analyzed using the Mann-Whitney test. To evaluate the prognostic relevance of the variables, probability was computed from the first day of taxane-based chemotherapy to the occurrence of disease progression, death, or last follow-up. Curves were made using the Kaplan-Meier method and the difference between the curves was analyzed using a log-rank test. Multivariate survival analysis was carried out using a logistic regression model. A P value of <0.05 was regarded as statistically significant.

Table 1. Primer sequences

Primer sequence of the *Bak* gene
Exon 2
S: 5′-CTGCTTTTTCTCGCCCTTCC-3′
AS: 5′-TGGTTATGGGATGGGTGAGG-3′
Exon 3
S: 5′-TGACTCCCAGCTTTGATCCT-3′
AS: 5′-TGCCTCCCTGAAGATGTCCT-3′
Exon 4
S: 5′-TCCCGACTGCCTGGTTACTG-3′
AS: 5′-GGCAGGGTATGGTATGGTTG-3′
Exon 5
S: 5′-CAATGCTATGGGATGCTCTG-3′
AS: 5′-CCAACAGCAGGGAGGACATT-3′
Exon 6
S: 5′-TGACCACCTTGTTTCTCCCG-3′
AS: 5′-GCAAGGGAACAGAGAAGGCA-3′
Primer sequence of the *Bax* gene
Exon 1
S: 5′-CGATCAGCGGGGCTCTCA-3′
AS: 5′-CAGGCCGGTAGGAAGCAT-3′
Exon 2
S: 5′-CCCCTAGAACCCAAGAGTC-3′
AS: 5′-CGGGGTGTCACTCCTC-3′
Exon 3
S: 5′-ATCCAGGATCGAGCAGGGCG-3′
AS: 5′-ACTCGCTCAGCTTCTTGGTG-3′
Exon 4
S: 5′-TCTCCCTGCAGGATGATTGC-3′
AS: 5′-TCCCCAGGTCCTCACAGA-3′
Exon 5
S: 5′-CAGGCAGTGGGGACAAGGTT-3′
AS: 5′-GCGGTGGTGGGGGTGAGGAG-3′
Exon 6
S: 5′-CCCCTGGCCGAGTCACTGAA-3′
AS: 5′-AATGCCCATGTCCCCCAATC-3′

Abbreviations: S, sense; AS, antisense.

Results

Expression of Bcl-2, Bak, or Bax in localized prostate cancer and HRPC samples. In 40 cases of localized prostate cancer, Bcl-2, Bak, and Bax expression was positive in 12 (30.0%), 31 (77.5%), and 38 (95.0%), respectively. As shown in Fig. 1A to C, Bcl-2, Bak, and Bax expression was typically found in the cytoplasm of prostate cancer cells. Bcl-2 protein was predominantly expressed in cancer cells of the basal layer, whereas Bak and Bax protein was diffuse and localized independent of cellular polarity (Fig. 1A-C). As shown in Fig. 1D, increased Bcl-2 expression was associated with higher Gleason score ($P < 0.05$). Likewise, a stepwise increase of Bcl-2 expression was found with advancing pT category ($P < 0.01$). Bak and Bax expression did not correlate with Gleason score or pT category. Dividing the 40 localized prostate cancer cases into two groups according to the median preoperative PSA level, Bax expression was higher in prostate cancer cases with lower preoperative serum PSA levels (under 8.95 ng/mL) than in those with higher levels (over 8.95 ng/mL; $P < 0.01$). There was no significant correlation between preoperative serum PSA level and either Bcl-2 or Bak expression. Additionally, no significant association was found between Bcl-2, Bak, or Bax protein expression and biochemical failure after prostatectomy.

Representative immunostaining of Bcl-2, Bak, and Bax in HRPC samples before treatment with taxane-based chemotherapy is shown in Fig. 2A to C. Among the 30 HRPC samples that showed viable cancer cells within the biopsy specimen before treatment with taxane-based chemotherapy, positive expression of Bcl-2, Bak, and Bax was observed in 18 (60.0%), 10 (33.3%), and 13 (43.3%), respectively. Furthermore, in the other 19 HRPC cases who were not exposed to taxane-based chemotherapy, positive expression of Bcl-2, Bak, and Bax was observed in 13 (68.4%), 6 (31.6%), and 3 (15.8%), respectively. There were no differences between Bcl-2, Bak, and Bax expression in HRPC with taxane-based chemotherapy and in HRPC without it. As shown in Fig. 2D, Bcl-2 expression was significantly higher in HRPC samples than in localized prostate cancer samples ($P < 0.02$), whereas Bak and Bax expression were significantly higher in localized prostate cancer samples than in HRPC samples ($P < 0.05$ and $P < 0.001$, respectively).

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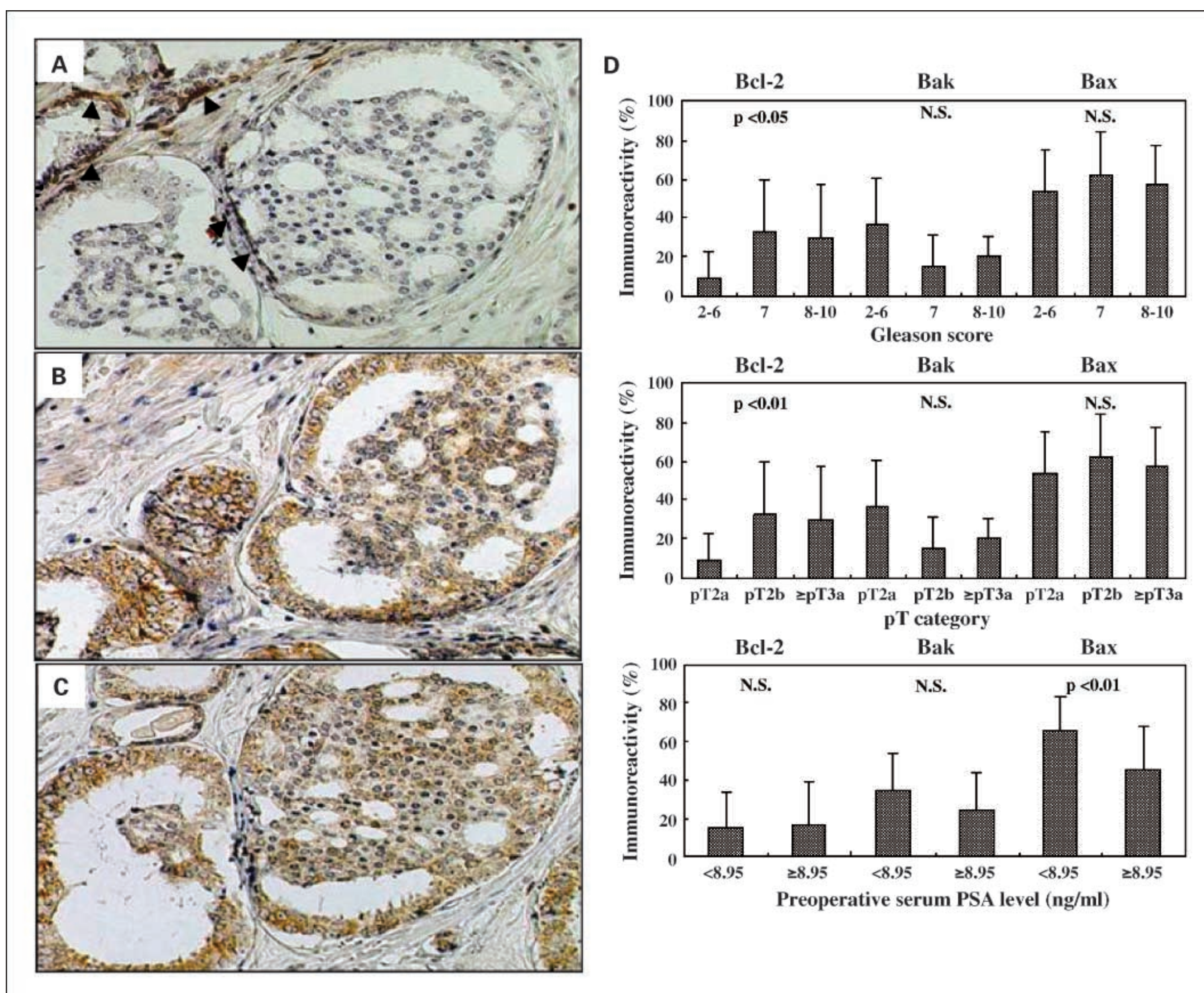

Fig. 1. Bcl-2, Bak, or Bax expression in localized prostate cancer and relationships with clinicopathologic findingsTypical immunostaining of Bcl-2 (*A*), Bak (*B*), and Bax (*C*) in a case of localized prostate cancer ($pT_{2a}N_0M_0$; Gleason, 4+3). Magnification, ×200. Bcl-2 expression is predominantly found in cancer cells of the basal layer (*A*; *arrowhead*). Bak and Bax expression are diffusely found in the cytoplasm of prostate cancer cells. *D*, relationship of Bcl-2, Bak, or Bax expression with Gleason score, pT category, or preoperative PSA level. Bcl-2 expression correlates with Gleason score and pT category ($P < 0.05$ and $P < 0.01$, respectively). Bax expression is significantly higher in cases with a lower preoperative serum PSA level than those with a higher PSA level ($P < 0.01$). N.S., not significant.

Expression of Bcl-2, Bak, or Bax and therapeutic response to taxane-based chemotherapy in HRPC. Individual HRPC data for therapeutic response to taxane-based chemotherapy are shown in Table 2. All cases showed decreased PSA levels (at nadir) in comparison with PSA levels at the starting point of chemotherapy (PSA at the baseline). The mean %PSA decrease by treatment with taxane-based chemotherapy was 84.3% ranging from 51.0% to 99.8%. No association was found between %PSA decrease by treatment with taxane-based chemotherapy and expression levels of Bcl-2, Bak, or Bax protein before treatment with chemotherapy (Fig. 3A). Sixteen of 30 cases with HRPC showed lymph node metastasis. The effect of chemotherapy on lymph node metastasis was partial response in nine cases and stable disease in seven cases. Complete response or progressive disease was not observed. As shown in Fig. 3A, partial response samples showed significantly higher Bcl-2 expression than stable disease samples ($P < 0.05$). Furthermore, in overall response, Bcl-2 expression was significantly higher in partial response samples than in stable disease samples ($P < 0.02$). On the other hand, a correlation between Bak, Bax expression, and response of lymph node metastasis or overall response to chemotherapy was not found. Pathologic response at the primary site did not show any significant association with expression of Bcl-2, Bak, or Bax protein.

Expression of Bcl-2, Bak, or Bax and prognosis in HRPC cases treated with taxane-based chemotherapy. Median follow-up, median progression-free survival time, and median cause-specific survival time was 79, 45, and 95 weeks, respectively. Progression-free survival was significantly longer in HRPC cases with Bax-positive expression than in those with Bax-negative expression (51 versus 34 weeks; $P < 0.02$; Fig. 3B). HRPC cases with lower baseline PSA levels also showed longer

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progression-free survival (70 versus 35 weeks; $P < 0.001$). HRPC cases with Bcl-2–positive expression showed longer cause-specific survival than those with Bcl-2–negative expression (137 versus 52 weeks; $P < 0.001$; Fig. 3B). Prior estramustine phosphate therapy was also related to shorter cause-specific survival (52 weeks with estramustine versus 107 weeks without estramustine; $P < 0.03$). Because the variables used in this study might be interrelated, multivariate analysis was done using a logistic regression model. As shown in Table 3, baseline PSA level was found to be an independent predictor for progression-free survival ($P < 0.01$), whereas Bcl-2 expression before treatment with taxane-based chemotherapy and prior estramustine phosphate therapy were considered independent predictors for cause-specific survival ($P < 0.01$ and $P < 0.05$, respectively). However, multivariate analysis revealed that Bax expression was not an independent predictor for progression-free survival in HRPC.

Additionally, among the 19 HRPC cases who were not exposed to taxane-based chemotherapy, we did not find that any marker was an independent predictor for either cause-specific survival or progression-free survival.

PCR-SSCP and direct DNA sequencing of the **Bak** *and* **Bax** *genes.* SSCP analysis of the *Bax* gene revealed that only one case (HRPC 9) of 30 HRPC samples showed an abnormal band shift. In this case, biopsy DNA samples from normal and cancerous areas at the time of prostate cancer diagnosis did not show any aberrant band shifts on SSCP gel; however, cancerous areas of HRPC samples did show abnormal band migration (Fig. 4). Direct DNA sequencing showed a single nucleotide substitution (G-to-A transition) in exon 3 of the *Bax* gene, leading to an amino acid alteration (Arg to His) at codon 65. In localized prostate cancer samples, no mutation of the *Bax* gene was detected. In the present series, there were no mutations of the *Bak* gene in either localized prostate cancer or HRPC samples.

Discussion

An impaired apoptotic pathway is thought to be a major contributing factor in the development of HRPC. Proapoptotic (Bak and Bax) and antiapoptotic (Bcl-2) molecules can form homodimers and heterodimers (2–5) and the relative ratios of these dimers are believed to regulate the sensitivity of cells toward either survival or apoptosis (6). Among those proteins related to apoptosis, up-regulation of Bcl-2 is frequently associated with poor prognosis in several cancers, including prostate cancer (8–11, 33). In this study, Bcl-2 expression

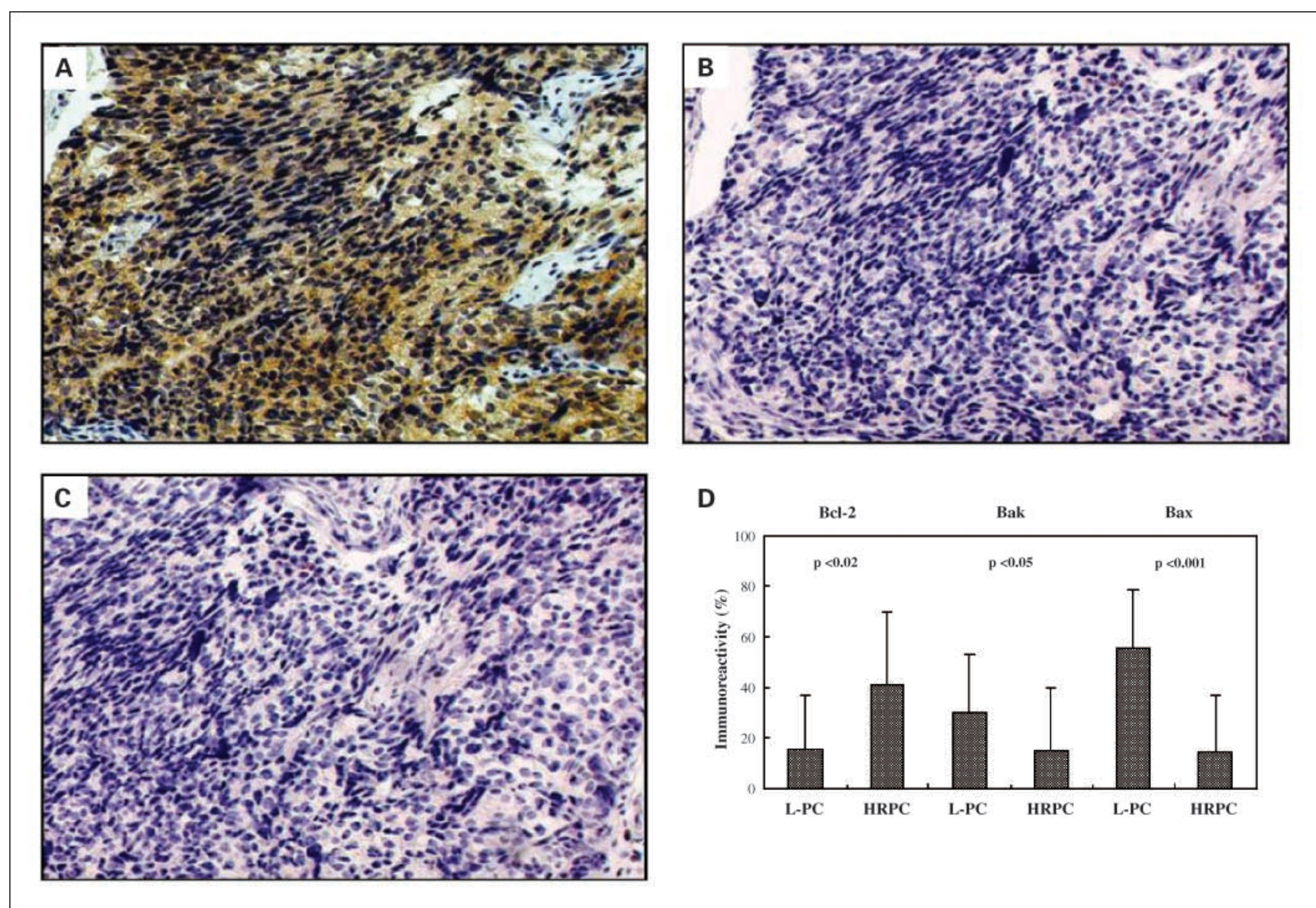

Fig. 2. Bcl-2, Bak, or Bax expression in HRPC and comparisons with localized prostate cancer. Typical immunostaining of Bcl-2 (*A*), Bak (*B*), and Bax (*C*) in the same HRPC tissue. Magnification, ×200. Note the diffuse distribution of cancer cells with strong Bcl-2 expression (*A*), and the scanty distribution of cancer cells with extremely weak expression of Bak (*B*) and Bax (*C*). *D,* Bcl-2 expression is significantly higher in HRPC than in localized prostate cancer (*L-PC*; $P < 0.02$), whereas Bak and Bax expression are significantly higher in localized prostate cancer than in HRPC ($P < 0.05$ and $P < 0.001$, respectively).

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Table 2. HRPC patients background and therapeutic response to taxane-based chemotherapy

Patient no.	Age (y)	Response to chemotherapy					
		PSA (% decrease)	Lymph node	Liver	Bone	Primary site	Overall response
1	78	90.6	(−)	(−)	SD	Response (+)	PR
2	64	88.3	(−)	(−)	SD	Response (+)	PR
3	78	85.1	PR	(−)	SD	Response (+)	PR
4	80	97.3	PR	(−)	(−)	Response (+)	PR
5	70	96.0	SD	(−)	SD	Response (+)	SD
6	69	99.8	(−)	(−)	SD	Response (+)	PR
7	75	95.5	SD	(−)	SD	Response (−)	PD
8	65	98.3	(−)	(−)	SD	No change	PR
9	76	90.2	(−)	(−)	SD	Response (+)	PR
10	66	99.3	(−)	(−)	SD	Response (+)	PR
11	80	99.3	(−)	(−)	SD	No change	PR
12	62	93.8	PR	(−)	SD	Response (+)	PR
13	63	87.0	SD	(−)	SD	Response (+)	SD
14	76	83.6	PR	(−)	SD	Response (+)	PR
15	72	81.9	(−)	(−)	(−)	Response (+)	PR
16	76	68.3	SD	(−)	SD	No change	SD
17	67	90.1	PR	PR	SD	Response (+)	PR
18	76	60.5	SD	(−)	PR	No change	SD
19	75	95.5	(−)	(−)	SD	Response (+)	PR
20	73	94.2	(−)	(−)	SD	Response (+)	PR
21	77	92.0	PR	(−)	SD	Response (+)	PR
22	73	55.9	SD	(−)	SD	Response (+)	SD
23	71	87.7	(−)	(−)	SD	No change	PR
24	65	97.2	PR	(−)	SD	Response (+)	PR
25	79	91.8	PR	(−)	(−)	Response (+)	PR
26	70	51.0	PR	(−)	SD	Response (+)	SD
27	72	56.0	(−)	(−)	SD	Response (+)	SD
28	69	52.0	(−)	(−)	(−)	Response (+)	SD
29	71	98.9	SD	(−)	SD	No change	PR
30	73	51.0	(−)	(−)	SD	Response (+)	SD

Abbreviations: CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; (−), no metastatic lesions detected.

was correlated with a higher Gleason score and pT category and was also higher in HRPC than in localized prostate cancer. This positive association of Bcl-2 expression with worse clinicopathologic characteristics is consistent with previous reports (8 – 11). Regarding proapoptotic molecules, reduced expression of Bak and Bax predicts a poor clinical outcome for several malignant tumors (30, 34). Our results showed that although Bak and Bax expression was not related to Gleason score or pT category, expression of both was significantly higher in localized prostate cancer than in HRPC. In addition, decreased Bax expression was associated with an increased preoperative PSA level in localized prostate cancer and early disease progression in HRPC. These findings suggest that up-regulation of antiapoptotic Bcl-2 protein or down-regulation of proapoptotic proteins may interact with the processes involved in the development of HRPC as well as disease progression in prostate cancer. Although the mechanisms underlying reduced expression of Bak and Bax protein in HRPC remain unclear, recent cDNA array analysis coupled with restriction landmark genomic scanning using multiple myeloma cells revealed that expression of *Bak* and *Bax* genes is probably down-regulated by promoter hypermethylation (35). It is possible that epigenetic regulation of the *Bak* and *Bax* genes is involved in the emergence of the more aggressive prostate cancer phenotype as well. In fact, our study showed that genetic alterations in the *Bak* and *Bax* genes are rare events in prostate cancer, including HRPC, in spite of their decreased expression in HRPC.

In vitro, phosphorylation of Bcl-2 and subsequent activation of apoptotic processes has been induced only in Bcl-2–expressing HRPC cells after treatment with taxanes, indicating that acceleration of apoptosis by taxanes can be recruited by the presence of Bcl-2 protein (22, 23). Based on these findings, we hypothesized that therapeutic response to taxane-based chemotherapy in HRPC might be predicted by the level of Bcl-2 expression before treatment with taxane-based chemotherapy, and Bcl-2–positive HRPC cases might have a better therapeutic response to taxane-based chemotherapy than Bcl-2–negative cases. In this study, HRPC cases showing partial response to chemotherapy in lymph node metastasis and overall response had a significantly increased level of Bcl-2 expression compared with HRPC showing stable disease. No significant association was found between Bcl-2 expression and pathologic response at the primary site to taxane-based chemotherapy, because in our series only one case showed an unfavorable response to chemotherapy (Table 2). As shown in Fig. 3B, a significantly longer cause-specific survival time was found in Bcl-2–positive HRPC cases than in Bcl-2–negative cases. In addition, multivariate analysis revealed that Bcl-2 expression was an independent predictor for cause-specific survival. Although higher PSA levels at baseline are related to early disease progression after chemotherapy, neither PSA levels at baseline nor PSA levels at nadir were related to therapeutic response to chemotherapy. Thus, Bcl-2 expression might be a reliable surrogate marker

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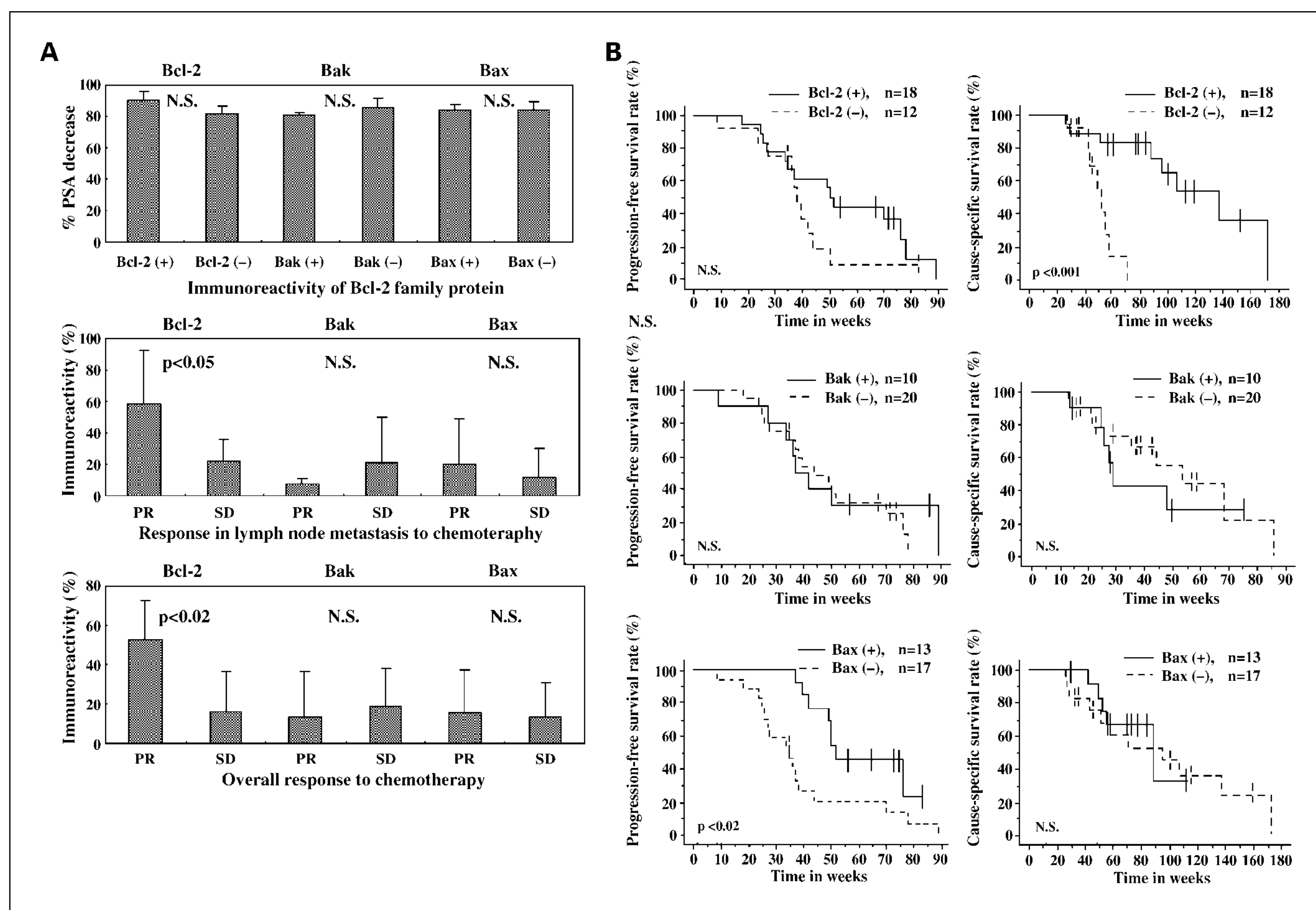

Fig. 3. Bcl-2, Bak, or Bax expression and therapeutic response to taxane-based chemotherapy and prognosis in HRPC. *A,* relationship of Bcl-2, Bak, or Bax expression with %PSA decrease, response in lymph node involvement, or overall response. Note that only Bcl-2 expression is significantly associated with response in lymph node metastasis and overall response ($P < 0.05$ and $P < 0.02$, respectively). *B,* relationship of Bcl-2, Bak, or Bax expression with progression-free survival or cause-specific survival probability. Note that Bcl-2 and Bax expression is significantly associated with cause-specific and progression-free survival, respectively ($P < 0.001$ and $P < 0.02$, respectively).

over the serum PSA level when predicting survival benefit in HRPC patients undergoing taxane-based chemotherapy. In other words, analysis of Bcl-2 expression before treatment in addition to PSA measurement could discriminate between HRPC patients who might benefit from taxane-based chemotherapy and those who might not. Furthermore, we have also evaluated an additional 19 HRPC cases who were not exposed to taxane-based chemotherapy as a control group. In this group, Bcl-2 expression was not an independent predictor for cause-specific survival. Consistent with our results, a previous report showed that Bcl-2 seemed not to be an independent predictor for survival of HRPC cases who were not exposed to taxane-based chemotherapy in multivariate analysis (36). Consequentially, when taxane-based chemotherapy is considered a first-line chemotherapy for HRPC, Bcl-2 expression analysis before treatment with taxane-based chemotherapy might identify HRPC cases who have a survival benefit from chemotherapy in advance.

Table 3. Multivariate analysis for progression-free survival and cause-specific survival

Variables	Variable estimate (SE)	Conditional risk ratio (95% confidence interval)	*P*
Progression-free survival			
Age (y)	0.336 (0.438)	1.399 (0.593-3.299)	Not significant
PSA at baseline	1.626 (0.498)	5.086 (1.916-13.501)	<0.01
Bax immunoreactivity	1.107 (0.450)	3.026 (1.251-7.317)	Not significant
Cause-specific survival			
Age (y)	0.576 (0.553)	1.780 (0.602-5.258)	Not significant
Prior estramustine phosphate therapy	1.282 (0.625)	3.602 (1.058-12.268)	<0.05
Bcl-2 immunoreactivity	2.218 (0.709)	9.188 (2.288-36.894)	<0.01

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Functionally, the proapoptotic effect of the Bak and Bax proteins is dependent on Bcl-2, because Bak and Bax proteins can heterodimerize with Bcl-2 protein and antagonize the functional role of Bcl-2 as an antiapoptotic molecule (2–5). Consistent with our results, up-regulation of Bcl-2 has been reported as frequent events in HRPC (8–11). However, Bcl-2 mutation has been considered an extremely rare event in human malignancies. Taking these into consideration, we focused on the mutational analysis of the *Bak* and *Bax* genes, and hypothesized that mutant forms of Bak or Bax protein would be unable to form a heterodimer complex with Bcl-2 and so could not inhibit the antiapoptotic function of Bcl-2. In the present study, neither *Bak* nor *Bax* gene mutation was found in localized prostate cancer, and only one missense mutation of the *Bax* gene was found in HRPC. As shown in Fig. 4, biopsy DNA samples at the time of initial prostate cancer diagnosis did not show any mutations; however, the cancerous areas of the HRPC sample did show a mutation. This missense mutation (from Arg to His) affects the BH3 domain, which plays an important role in forming the Bax-Bax homodimer or Bax-Bcl-2 heterodimer (4). It is quite plausible therefore that the *Bax* gene mutation in the BH3 domain might affect the pattern of protein dimerization, leading to functional loss of Bax-mediated, apoptosis-promoting activity (14). Interestingly, because only DNA from HRPC tissue, but not that from either normal prostate or prostate cancer tissue before the development of HRPC, showed the *Bax* gene mutation (Fig. 4), this genetic alteration may be a late event and might have been involved in the development of HRPC.

To summarize, up-regulated Bcl-2 protein is associated with aggressive biological features of individual prostate cancers and appears to be associated with the development of HRPC. However, the presence of Bcl-2 protein, in turn, could indicate a survival benefit in HRPC patients scheduled for taxane-based chemotherapy. Further investigation based on this pilot study will hopefully lead to better clarification of the possible application of Bcl-2 expression as a predictive marker of HRPC treated with taxane-based chemotherapy.

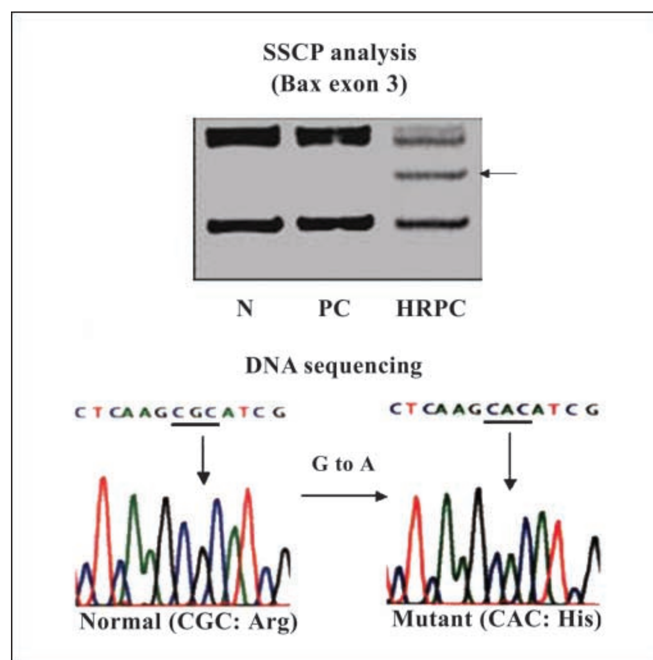

Fig. 4. Result of SSCP analysis and direct DNA sequencing in one HRPC case. Biopsy DNA samples from normal (*N*) and prostate cancer areas (*PC*) at the initial diagnosis of prostate cancer show no abnormal bands on SSCP gel; however, cancerous areas of the HRPC sample show abnormal band migration (*arrow*). Direct DNA sequencing reveals that a G-to-A transition occurred in exon 3 of the *Bax* gene, leading to an amino acid change of Arg (CGC) to His (CAC) at codon 65. Primer sequences of the *Bak* and *Bax* genes used in this study are shown in Table 1.

Although genetic alterations of proapoptotic genes, such as *Bak* and *Bax*, appear to be rare in human prostate cancer, prognostic relevance of reduced Bax expression with reference to early disease progression was found in HRPC. Other mechanisms, such as epigenetic promoter methylation, except for genetic alteration, might be involved in the down-regulation and/or functional loss of Bak or Bax protein in prostate cancer.

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