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Immunohistochemical Expression of Bcl2 is an Independent Predictor of Time-to-biochemical Failure in Patients with Clinically Localized Prostate Cancer Following Radical Prostatectomy

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Abstract. *Background: Whether the immunohistochemical expression (IHCE) of the bcl2, the p53 and of the prostate apoptosis response-4 (PAR4) proteins is associated with pre-operative PSA levels, post-operative parameters of prostate cancer (PC) pathology, surgical staging or biochemical failure (BF) of patients with clinically localized PC who underwent radical prostatectomy (RP) of curative intent, was investigated. Patients and Methods: A retrospective analysis of clinical data evaluating surgical specimens of 131 patients with PC, consecutively treated with RP for clinically localized disease, was performed. The IHC method of streptavidin biotin peroxidase on paraffin tissue sections was used to detect bcl2 and p53 oncoproteins and PAR4 pro-apoptotic protein expression in surgical specimens. Results: Statistically significant relationships were detected between: (i) p53 IHC expression and infiltration of periprostatic tissue (IPT; p=0.011); (ii) tumor volume (TV; p=0.027); and (iii) bcl2 IHCE and absence of prostatic intraepithelial neoplasia (PIN) (p=0.004). Biochemical failure (BF) was documented in 37% of these patients. Kaplan-Meier survival curves showed that the IHCE of bcl2 and p53 was significantly related to BF. Taking the hazard ratio (HR) estimated from the Cox proportional hazard regression model to be 1.00 for patients with negative bcl2 IHCE, a value of 2.82 was found for patients with positive bcl2 IHCE (p=0.015, 95% CI=1.22-6.47). The HR for patients with positive p53 IHCE was 2.05 (p=0.048, 95% CI=1.00-4.19). Multivariate analysis showed that only seminal vesicle invasion (SVI), pelvic lymph node metastasis (PLNM) and bcl2 IHCE were independent predictors for BF (HR=3.06, 3.31 and 3.15; p=0.048, p=0.031 and p=0.031 for SVI, PLNM and bcl2 IHCE, respectively). Conclusion: Bcl2 immunohistochemical overexpression in specimens of RP suggests high risk for BF in clinically localized PC.*

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The biological behavior of prostate cancer (PC) tumors harbored by patients with clinically localized disease varies between latent slow-growing tumors and highly aggressive tumors, consisting of cancer cells with metastatic potential. Radical prostatectomy (RP) is the primary treatment of curative intent for patients diagnosed with clinically localized PC. However, there are a large number of surgically treated patients in whom PC already harbors occult tumor extensions either to the extraprostatic tissue or to distal organs, lymph nodes and bones, which are apparently responsible for disease recurrence after RP (1, 2). Tumor grade, clinical stage and pre-treatment prostate-specific antigen (PSA) are often used as prognostic markers for the presence of extraprostatic disease and the estimation of risk for biochemical failure (BF) after RP, but none of them has the power as a single prognostic factor to predict the presence of extraprostatic disease and time-to-BF (3).

In theory, pre-operative and post-operative management decisions for patients with PC should ideally depend on an accurate assessment of tumor aggressiveness/metastatic potential and a safe diagnostic test detecting the presence of extraprostatic disease and micro metastasis. None is available. Recently, pre-operative and post-operative molecular staging, using reverse transcriptase-polymerase chain reaction (RT-PCR) to detect PSA and prostate-

specific membrane antigen (PSMA) transcripts in peripheral blood and bone marrow biopsy have shown encouraging correlations between the positive detection of these transcripts with time-to-BF after RP in patients with clinically localized PC (4-6). Nevertheless, this method is still far from being widely accepted or routinely employed (7).

In the present study, the immunohistochemical expression (IHCE) of bcl2, p53 and prostate apoptosis response-4 (PAR4) protein was examined in RP specimens of 131 patients with PC, consecutively treated with RP for clinically localized disease in the Urology Clinic of "Evangelismos" General Hospital in Athens, Greece, from 1-1-1994 to 12-12-2001. We report the correlation between the above markers and several pathological and clinical parameters of these PC patients, including BF after RP of curative intent.

Patients and Methods

The clinical-follow up data were analyzed retrospectively, and the surgical specimens of 131 patients (ages ranging from 47 to 76 years, median of 66) with newly diagnosed PC were reviewed. After being clinically diagnosed with localized disease, these patients had undergone RP and pelvic lymph node resection, as consecutively performed in "Evangelismos" General Hospital, Athens, Greece, between January 1994 and December 2001. Pre-operative serum PSA concentrations ranged from 2.5 to 45.0 ng/ml with a median of 9.23 ng/ml. Pre-operative transrectal ultrasonography guided-biopsy (TRUS-B) material was available in 83 cases for re-evaluation by our expert pathologist, while for all TRUS-B, a copy of the official pathology report with the original diagnosis from a certified pathologist (patients diagnosed in other institutions and referred to us for surgery) was kept on record. Follow-up information (median follow-up period 28 months) was available for 94 patients. The remainder continued their follow-up in other hospitals and medical units, as most of them lived in the provinces far from Athens. PSA levels above 0.2 ng/ml, on at least two occasions in a 2-month period, defined BF.

RP specimens were submitted fresh for routine histopathological examination. These were weighed, stained and fixed in buffer-formalin. The prostate weight ranged from 8 to 250 grams, with a median of 45 grams. Routine processing included sectioning and paraffin embedding of the whole prostate gland in 4-mm-thick paraffin sections used for haematoxylin-eosin and IHC staining. Almost all selected sections contained benign prostatic glands, which served as internal positive controls for bcl2 and PAR4. Tumor volume (percentage) referred to the percentage of prostate volume involved by PC. Grading was established according to the Gleason score (GS) differentiation system (range 2-10) (8). GS 7 carcinomas were stratified to 7a (3+4) and 7b (4+3), according to the predominant grade, as there is increasing evidence that the biology of 7a is different from 7b and can affect prognosis (9, 10). A 2-scale grouping was used for the final evaluation of the histological grading, as follows: group I <7a and group II >7b. The area with the highest GS was a selected IHC of RP specimen. High-grade prostatic intraepithelial neoplasia (PIN) was also evaluated. Staging was carried out according to the TNM 2002 classification (11). The following pathological parameters were evaluated: extension of the tumor to stained specimen margin, extracapsular extension into periprostatic tissue, seminal vesicle invasion (SVI) and pelvic lymph node metastasis (PLNM).

Table I. *Pathological characteristics of 131 radical prostatectomies performed on patients with localized prostate cancer.*

Variable (No. of RP[a] specimens)	RP specimens (%)
Gleason Score (130)	
5	2 (1)
6	27 (21)
7a	32 (25)
7b	43 (33)
8	16 (12)
9	10 (8)
x	1
Surgical Margins (130)	
Negative	86 (66)
Positive	44 (34)
x	1
PIN[b] (131)	
Absent	28 (21)
Present	103 (79)
Periprostatic Tissue Invasion (130)	
Absent	60 (46)
Present	70 (54)
x	1
Seminal Vesicle Invasion (130)	
Absent	96 (74)
Present	34 (26)
x	1
Nodal Status (130)	
Negative	120 (92)
Positive	10 (8)
x	1

[a]Radical prostatectomy;
[b]Prostatic Intraepithelial Neoplasia;
x. Status unknown.

Immunohistochemistry. The streptavidin biotin peroxidase protocol using the DAKO LSAB+Kit Peroxidase was performed, with commercially available antibodies: mouse monoclonal antibody against bcl2 (clone 124; Dako, Glostrup, Denmark), at a 1:20 dilution, mouse monoclonal antibody against p53 (clone Pab240; Dako), at a 1:50 dilution, and rabbit polyclonal antibody PAR4 (clone A-10: sc-1666, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), at a 1:50 dilution. Staining procedures included de-paraffinization in warm xylene for 5 min with two changes of xylene at room temperature, followed by rehydration by transfer through graded alcohols. Endogenous peroxidase activity was blocked with 0.5% H_20_2 in methanol for 10 min. The sections were pre-treated with 10 mmol/L citrate buffer (pH 6.1) in a microwave for 5 min and incubated overnight at 4˚C with the hK10 primary rabbit polyclonal and mouse monoclonal antibodies in 3% BSA. After two washes of the sections in 50 mM Tris buffer (pH 7.6), the biotinylated Link (DAKO Corporation, USA) was applied for 15 min, and a streptavidin-peroxidase conjugate followed for another 15 min. The enzymatic reaction was developed in a freshly

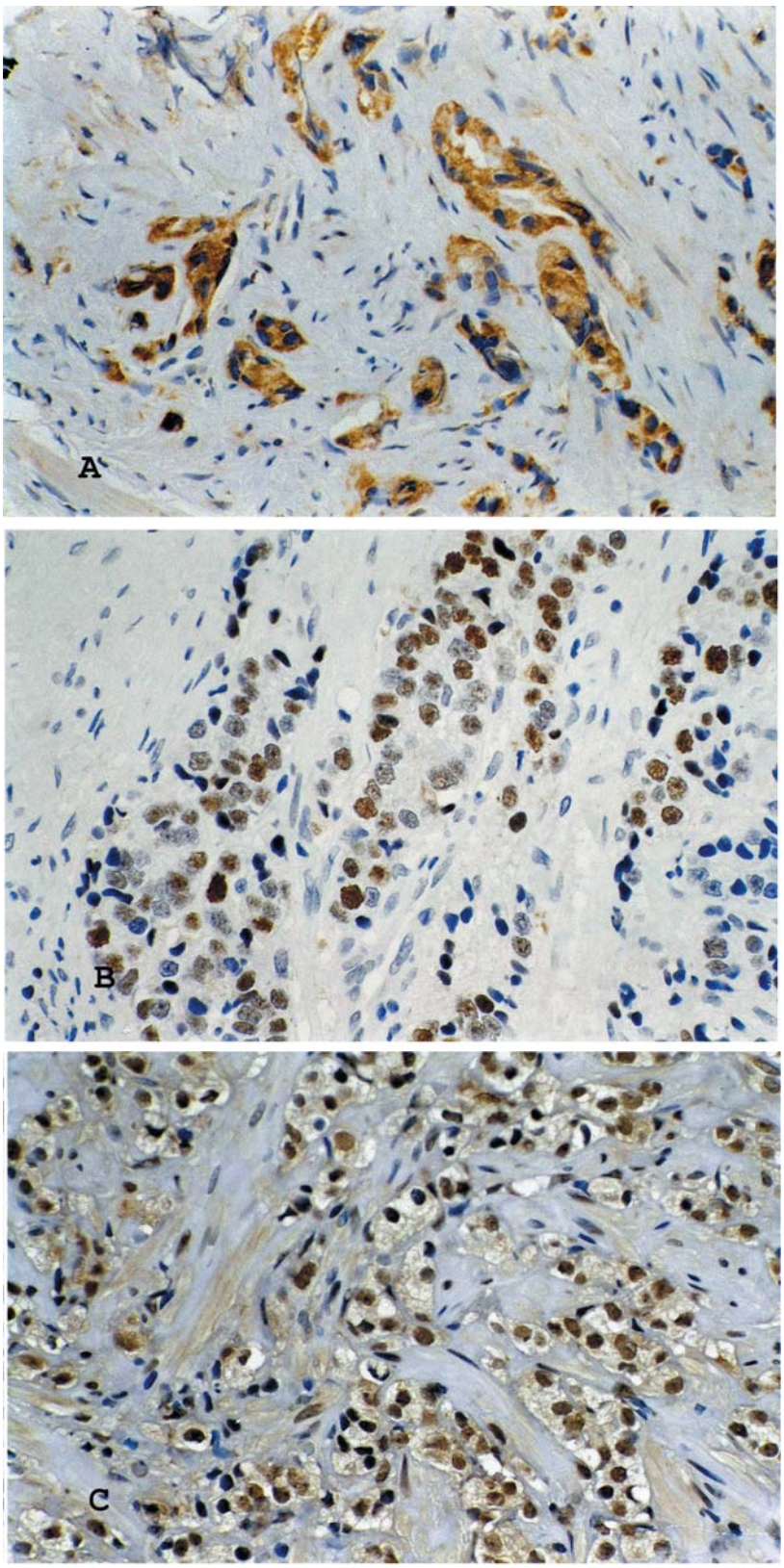

Figure 1. *A: Diffuse and extensive cytoplasmic bcl2 immunohistochemical expression (IHCE) in prostate cancer (PC), B: High nuclear p53 IHCE in PC, C: Extensive nuclear and cytoplasmic PAR4 IHCE in PC. Magnification x 200.*

Table II. *Relationship between bcl2 status and other variables in 131 radical prostatectomy specimens of prostate cancer.*

Variable	No. of patients (%)			
	Patients	bcl2-negative	bcl2-positive	*p* value[a]
Gleason Score				
≤7a	61	56 (91.8)	5 (8.2)	0.77
7b-10	69	62 (89.9)	7 (10.1)	
x	1			
Surgical Margins				
Negative	86	78 (90.7)	8 (9.3)	0.99
Positive	44	40 (90.9)	4 (9.1)	
x	1			
PIN[b]				
Absent	28	21 (75.0)	7 (25.0)	0.004
Present	103	98 (95.1)	5 (4.9)	
Periprostatic				
Tissue Invasion				
Absent	60	54 (90.0)	6 (10.0)	0.99
Present	70	64 (91.4)	6 (8.6)	
x	1			
Seminal				
Vesicle Invasion				
Absent	96	87 (90.6)	9 (9.4)	0.99
Present	34	31 (91.2)	3 (8.8)	
x	1			
Nodal Status				
Negative	120	109 (90.8)	11 (9.2)	0.99
Positive	10	9 (90.0)	1 (10.0)	
x	1			
PAR4 Status				
Negative	68	60 (88.2)	8 (11.8)	0.37
Positive	63	59 (93.7)	4 (6.3)	

[a]Fisher's Exact Test;
[b]Prostatic Intraepithelial Neoplasia;
x. Status unknown.

Table III. *Relationship between p53 status and other variables in 131 radical prostatectomy specimens with prostate cancer.*

Variable	No. of patients (%)			
	Patients	p53 low	p53 high	*p* value[a]
Gleason Score				
≤7a	61	50 (82.0)	11 (18.0)	0.99
7b-10	69	57 (82.6)	12 (17.4)	
x	1			
Surgical Margins				
Negative	86	71 (82.6)	15 (17.4)	0.99
Positive	44	36 (81.8)	8 (18.2)	
x	1			
PIN[b]				
Absent	28	22 (78.6)	6 (21.4)	0.58
Present	103	86 (83.5)	17 (16.5)	
Periprostatic				
Tissue Invasion				
Absent	60	55 (91.7)	5 (8.3)	0.011
Present	70	52 (74.3)	18 (25.7)	
x	1			
Seminal				
Vesicle Invasion				
Absent	96	81 (84.4)	15 (15.6)	0.31
Present	34	26 (76.5)	8 (23.5)	
x	1			
Nodal Status				
Negative	120	100 (83.3)	20 (16.7)	0.38
Positive	10	7 (70.0)	3 (30.0)	
x	1			
Bcl2 Status				
Negative	119	100 (84.0)	19 (16.0)	0.22
Positive	12	8 (66.7)	4 (33.3)	
PAR4 Status				
Negative	68	57 (83.8)	11 (16.2)	0.82
Positive	63	51 (81.0)	12 (19.0)	

[a]Fisher's Exact Test;
[b]Prostatic Intraepithelial Neoplasia;
x. Status unknown.

prepared solution of 3, 3'-diaminobenzidine tetra hydrochloride using DAKO Liquid DAB Substrate-Chromogen Solution for 10 min (brown color). The sections were then counterstained with haemalum, dehydrated, cleared in xylene and mounted. For bcl2 and PAR4, internal positive controls were available. For p53, colon carcinoma with known p53 immunoexpression was used as a positive control.

Tissue markers scoring system. Most of the staining was heterogeneous and focal in nature. In the area of strongest staining, 1000 consecutive malignant cells were counted. The number of cells with positive cytoplasmic staining for bcl2 and nuclear staining for p53 were recorded. For PAR4, either cytoplasmic or nuclear staining was regarded as positive. The results were then grouped. The extension of cytoplasmic staining for bcl2 in tumor areas was categorized as negative=negative staining or focal areas of staining in a percentage up to 10%, and as positive=focal or diffuse staining (>10%). Nuclear p53 accumulation was categorized as negative=negative staining or scattered positive cells in a percentage <2% without clustering of positive cells, and as positive=>2% positive cells or <2% with clustering, as described by Quinn *et al.* (12). Accordingly, PAR4 immunostainining was classified as negative=negative staining or focal staining in a percentage up to 10% and as positive=focal or diffuse staining (>10%).

Statistics. For analysis of data, the patients were subdivided into groups based on different clinical or pathological parameters. In this analysis, p53, bcl2 and PAR4 were classified into two categories (positive and negative groups), and the associations between gene expression status and other qualitative variables were analyzed using the Fisher's Exact test. Because the distribution of serum PSA, tumor percentage, prostate weight and patients' age were not Gaussian, the analysis of differences in p53, bcl2 and

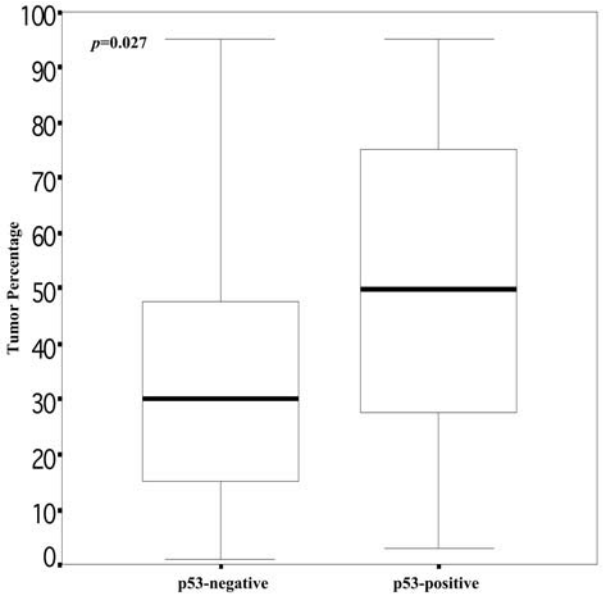

Figure 2. *Relationship between p53 IHCE and tumor volume in 131 radical prostatectomy specimens with prostate cancer.*

PAR4 values between two groups was performed with the nonparametric Mann-Whitney *U*-test.

Survival analyses were performed by constructing Kaplan-Meier Progression Free Survival (PFS) curves, where differences between curves were evaluated by the log-rank test, as well as by estimating the relative risks for relapse and death using the Cox proportional hazards regression model. Only patients for whom the status of all variables was known were included in the multivariate regression models, which incorporated p53, bcl2, PAR4 and all other variables for which the patients were characterized. In the multivariate analysis, the clinical and pathological variables that may affect progression-free survival, including GS, surgical margins, PIN, extra capsular periprostatic tissue invasion, seminal vesicle invasion, nodal status and tumor percentage were adjusted. Statistical analysis was performed using the SAS software (SAS Institute, Cary, NC, USA).

Results

Among the 94 patients for whom follow-up data were available, 35 (37%) presented BF. Prostate weight ranged between 8 g and 250 g (median: 45 g), and PC tumor volume ranged between 1% and 100% (median: 30%) of the prostate volume. Clinicopathological characteristics of the 131 RP specimens analyzed in this study are provided in Table I. Pre-operative GS on transrectal ultrasonography-guided biopsies (TRUS-B) generally agreed with this computed after RP. However, pre-operative GS was lower in 36% and higher in 7% from this estimated on RP specimens. Post-operative GS was taken into account in all steps of the study. In 61/131 specimens (47%), GS was estimated as ≤7a and in 69/131 (53%) as ≥7b (one missing case). In the RP specimens, the presence of positive surgical margins (34%), seminal vesicle involvement (26%), invasion of periprostatic tissue (54%) and pelvic lymph node involvement (8%) were found. High grade PIN was observed in 79% of the RP specimens.

Table IV. *Relationship between PAR4 status and other variables in 131 radical prostatectomy specimens with prostate cancer.*

Variable	No. of patients (%)			
	Patients	PAR4-negative	PAR4-positive	*p* value[a]
Gleason Score				
≤7a	61	34 (55.7)	27 (44.3)	0.48
7b-10	69	34 (49.3)	35 (50.7)	
x	1			
Surgical Margins				
Negative	86	47 (54.7)	39 (45.3)	0.46
Positive	44	21 (47.7)	23 (52.3)	
x	1			
PIN[b]				
Absent	28	14 (50.0)	14 (50.0)	0.83
Present	103	54 (52.4)	49 (47.6)	
Periprostatic Tissue Invasion				
Absent	60	28 (46.7)	32 (53.3)	0.29
Present	70	40 (57.1)	30 (42.9)	
x	1			
Seminal Vesicle Invasion				
Absent	96	51 (53.1)	45 (46.9)	0.84
Present	34	17 (50.0)	17 (50.0)	
x	1			
Nodal Status				
Negative	120	62 (51.7)	58 (48.3)	0.74
Positive	10	6 (60.0)	4 (40.0)	
x	1			

[a]Fisher's Exact Test;
[b]Prostatic Intraepithelial Neoplasia;
x. Status unknown.

Bcl2, PAR4 and p53 IHC expression. In the benign prostatic tissue, basal cells expressed bcl2, and PAR4 was expressed in both basal and secretory cells. Benign prostatic tissue was negative for p53. Fifty-two (40%) RP specimens exhibited some degree of bcl2 cytoplasmic expression. However, only 12/131 (9%) contained bcl2 expression of a diffuse pattern involving more than 10% of cancer cells (positive staining). In addition, low levels of p53 expression were detected in 73 out of 131 (56%) RP specimens. It should be noted that p53 IHC expression in less than 2% of tumor cells and without positive cell clustering was regarded as negative. Consequently, only 23/131 (18%) specimens were defined

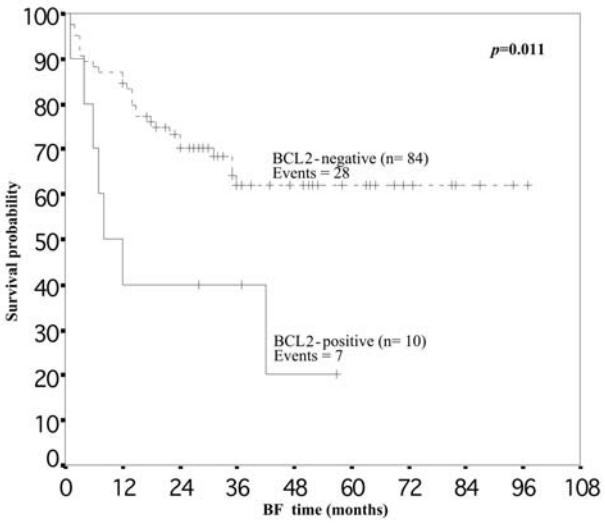

Figure 3. *Relationship between bcl2 immunoexpression and biochemical failure in 94 patients after radical prostatectomy specimens for localized prostate cancer.*

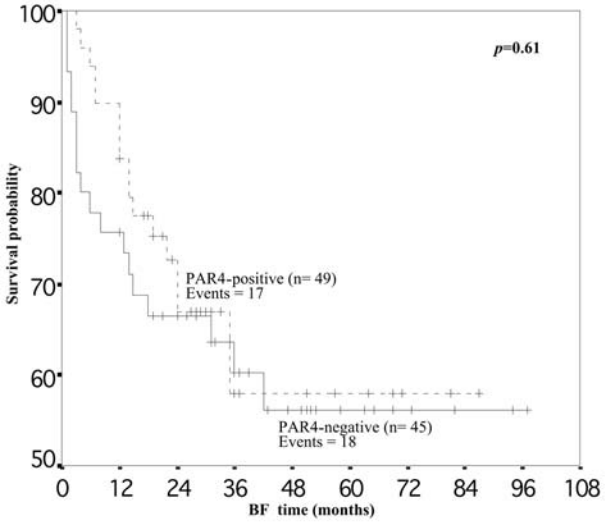

Figure 5. *Relationship between PAR4 immunoexpression and biochemical failure in 94 patients after radical prostatectomy for localized prostate cancer.*

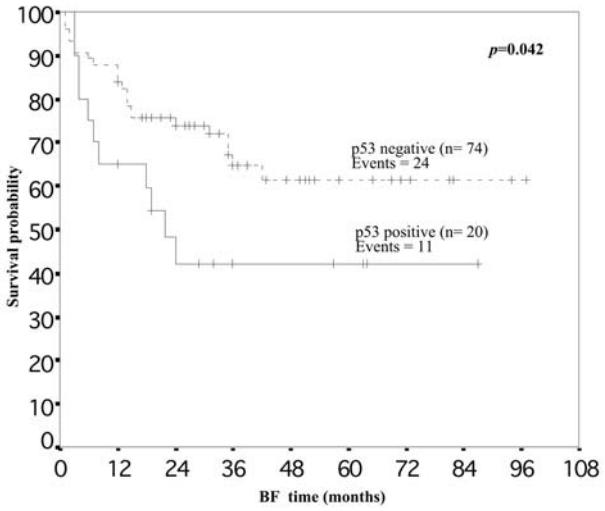

Figure 4. *Relationship between p53 immunoexpression and biochemical failure in 94 patients after radical prostatectomy for localized prostate cancer.*

as positive for p53 expression. Analysis of the PAR4 IHC expression showed that 111/131 (85%) of the RP specimens expressed PAR4 at low levels, but a diffuse or strong focal expression in 10% or more of the section was observed only in 62/131 (48%) cases (Figure 1A, B, C).

Bcl2, PAR4, p53 IHC expression vs. clinicopathological parameters. Bcl2 expression was not found to be related to GS, invasion of periprostatic tissue, seminal vesicle involvement, PLNM or positive surgical margins. Furthermore, there was no association of bcl2 expression with tumor volume and prostate weight. However, there was a significant relationship between bcl2 expression and the absence of PIN in the RP specimen (Table II).

IHC detection of nuclear p53 expression did show a significant positive relationship with periprostatic tissue invasion ($p=0.011$) and tumor percentage ($p=0.027$) in RP specimens. However, p53 expression did not show any significant correlation with GS, seminal vesicle involvement, PLNM, positive surgical margins, or prostate weight (Table III, Figure 2).

In addition, PAR4 HIC expression was not associated with GS, invasion of periprostatic tissue, seminal vesicles involvement, positive surgical margins, tumor volume, PIN, or prostate weight (Table IV).

Possible associations in IHC expression among the studied biomarkers were also examined, but no statistically significant correlation was found (Tables II and III). Furthermore, no statistically significant relationships between p53, bcl2, PAR4 and pre-operative serum PSA, patient's age or prostate weight were observed.

Prognostic value of bcl2, p53 and PAR4 expression for BF. Univariate analysis of bcl2 expression (more than 10% of tumor cells) and of p53 nuclear accumulation (more than 2% of tumor cells or clustering) detected an association of both IHCE with BF of the disease. Taking the hazard ratio (HR), estimated from a Cox proportional hazard regression model, to be 1.00 for patients with negative bcl2, a value of

Table V. *Univariate analysis of p53, bcl2, PAR4 and other variables with regard to BF.*

Variable	HR[a]	95% CI[b]	*p* value
P53			
Negative	1.00		
Positive	2.05	1.00-4.19	0.048
Bcl2			
Negative	1.00		
Positive	2.82	1.22-6.47	0.015
PAR4			
Negative	1.00		
Positive	0.94	0.76-1.17	0.61
Gleason Score			
≤7a	1.00		
7b-10	2.07	1.01-4.24	0.046
Surgical Margins			
Negative	1.00		
Positive	1.59	0.81-3.11	0.17
PIN			
Absent	1.00		
Present	0.72	0.34-1.51	0.72
Periprostatic Tissue Invasion			
Absent	1.00		
Present	3.23	1.51-6.91	0.002
Seminal Vesicle Invasion			
Absent	1.00		
Present	3.22	1.65-6.29	0.001
Nodal Status			
Negative	1.00		
Positive	3.76	1.42-9.94	0.008
Tumor Percentage	1.02	1.013-1.035	<0.001

[a]Hazard ratio (HR) estimated from Cox proportional hazard regression model;
[b]Confidence interval of the estimated HR.

Table VI. *Multivariate analysis of p53, bcl2, PAR4 and other variables with regard to BF.*

Variable	HR[a]	95% CI[b]	*p* value
P53			
Negative	1.00		
Positive	0.86	0.36-2.04	0.74
Bcl2			
Negative	1.00		
Positive	3.15	1.11-8.94	0.031
PAR4			
Negative	1.00		
Positive	1.05	0.81-1.36	0.69
Gleason Score			
≤7a	1.00		
7b-10	1.04	0.43-2.51	0.91
Surgical Margins			
Negative	1.00		
Positive	0.40	0.15-1.04	0.061
PIN			
Absent	1.00		
Present	1.12	0.47-2.69	0.79
Periprostatic Tissue Invasion			
Absent	1.00		
Present	3.06	1.01-9.25	0.048
Seminal Vesicle Invasion			
Absent	1.00		
Present	1.89	0.64-5.57	0.24
Nodal Status			
Negative	1.00		
Positive	3.31	3.31-1.11	0.031
Tumor Percentage	1.01	0.98-1.06	0.11

[a]Hazard ratio (HR) estimated from Cox proportional hazard regression model;
[b]Confidence interval of the estimated HR.

2.82 was calculated for patients with positive bcl2 ($p=0.015$, 95% CI=1.22-6.47). The HR was found to be 2.05 for patients with positive p53 ($p=0.048$, 95% CI=1.00-4.19) (Table IV, Figures 3 and 4). Among the 94 patients with follow-up, high levels of p53 expression were found in 20, and 11 of these patients relapsed (55%). In contrast, from the low p53 expression group only 24 (32%) patients relapsed. Accordingly, high bcl2 expression was found in 10 cases, of which 7 (70%) suffered recurrent disease. On the other hand, from the 84 patients with low bcl2 expression, biochemical relapse was identified in only 28 (33%). PAR4 expression did not show any correlation with clinical outcome (Table IV, Figure 5).

Univariate Cox regression analysis showed that the histological grade of RP specimens, the invasion of periprostatic tissue, seminal vesicle involvement, PLNM and tumor percentage were, as expected, strong predictors for BF after RP. However, positive surgical margins did not correlate significantly with BF. Furthermore, coexistence of high grade PIN in the specimen did not carry any prognostic significance (Table V). Multivariate analysis of parameters evaluated in this cohort study revealed that infiltration of periprostatic tissue, metastatic lymph node involvement and bcl2 IHC expression were independent prognostic factors for BF ($p=0.048$, $p=0.031$ and $p=0.031$; HR=3.06, HR=3.31, HR=3.15, respectively) (Table VI).

Discussion

PC is still the most commonly diagnosed cancer with significant lethality and morbidity. Over the past two decades, many studies have focused on the identification of prognostic markers that could predict which patients with localized disease would have recurrent disease after RP.

Conventional variables such as preoperative PSA, grade, stage and surgical margins are not able to predict outcome in an individual patient. Elucidation of such prognostic tissue markers would help the clinician to distinguish tumors with biologically aggressive behavior and such patients would define the high-risk group for disease recurrence after RP. Therefore, these patients would be candidates for adjuvant radiotherapy or adjuvant hormone ablation therapy (13-15).

Many molecular tissue markers have been studied for their immunohistochemical expression in PC, but to date no marker has been approved for routine assessment. Some markers such as p53, bcl2, Ki-67 (MIB1) and p27 expression as well as neovascularity assessment hold promise, but prospective multicenter studies are needed to confirm their prognostic significance. According to Dunsmuir *et al.* (16), the ideal IHC tissue marker would be expressed only in tumors with an aggressive phenotype. The expression should not be limited to parts of the heterogeneous tumor mix and adjacent less significant areas would also harbor the same information. The expression of this marker should be detected from the primary site and be independent of histological grade and other clinical variables such as stage and PSA levels. Laboratory detection of the biomarker should be consistent and inexpensive. With these presuppositions, an easy-to-understand and quantifiable risk estimate would be available for clinicians and patients.

The p53 tumor-suppressor gene, which has been very well studied in different tumors, is located on the short arm of chromosome 17 (17). It has a wide range of functions involved in apoptosis, cell cycle regulation and DNA replication (18-20). Mutation of the *p53* gene is the most frequently reported mutation in human cancer (19). However, there is still a discrepancy as to the frequency of *p53* mutations in PC, and as to their prognostic role (21-26). Over-expression of p53 protein has been investigated in a large number of different malignancies for its potential value as a prognostic marker. In PC, p53 expression has been correlated with mutations, which cause an increase in its half-life period and give an opportunity for IHC detection. Some studies have shown positive staining for p53 in PIN lesions, underlining the hypothesis that *p53* mutations may occur in pre-invasive lesions, which could progress into aggressive PCs (27-29). In contrast, other studies have shown p53 IHC expression to be confined to PC, especially in higher grades, and absent in BPH, PIN and adenosis (30-32). Studies of p53 assessment in prostate needle biopsy have been conflicting (33-39), as a result of tumor heterogeneity and multifocality. In contrast, p53 IHC expression in RP specimens has shown promising results. In previous studies with a large number of patients (ranging from 72 to 263) and decent follow-up, the p53 status was found to be an independent predictor of recurrence or disease-specific survival (12, 21, 40-42). In other studies, p53 expression was a statistically significant predictor of relapse in univariate analysis but not in multivariate, which included clinicopathological parameters or other tissue markers (31, 39). Negative results have also been reported. Uzoaru *et al.*, in a study of 134 PC specimens, did not find any prognostic significance for p53 expression (43). Increased p53 nuclear protein IHC expression appears to correlate with androgen independence (23), especially when accompanied by detection of bcl2 over-expression (3). Reported *p53* mutation rates vary widely, from only 4% (14) to 79% (44). Mirchandani *et al.* showed that within RP specimens there was widespread heterogeneity of *p53* mutations from tumor to tumor and within single tumors, and they proposed that this may contribute to the variation of results (45). Several reports have also confirmed that nuclear p53 accumulation is usually identified in locally regionally-advanced PC, in generalized disease and is associated with high grade (3, 21, 40, 46-49), advanced stage (3, 21, 40, 46) and resistance to hormonal treatment (3, 49). Mutations of *p53* are considered to be a late event in prostatic carcinogenesis (50).

In addition, the bcl2 family is a group of genes that encode for structurally-related proteins acting in the apoptosis procedure either as inhibitors (bcl2, bcl-xl, mcl-1, bcl-w) or as suppressors (bax, bak, bik, bad, bcl-xs) (51). The bcl2 protein protects cells from going into apoptosis, and in normal and hyperplastic prostatic glands it is expressed in the cytoplasm of basal epithelial cells. Over-expression of bcl2 due to a transforming mutation has been associated with carcinogenesis. Over-expression of bcl2 is seen in a lower percentage of primary tumors than p53 expression, but the reported rates vary significantly, with a range between 4% and 52% (3, 21, 40, 52). In previous studies a correlation between bcl2 over-expression and higher stage (40), GS and nuclear grade was reported (21). McDonnell *et al.* (53) demonstrated a strong association between bcl2 IHC over-expression and hormone therapy resistance. Previous studies have suggested that hormone-refractory prostatic carcinomas characteristically present diffuse expression of bcl2 protein throughout the tumor (53). In localized PC treated by RP, bcl2 immunohistochemical (IHC) expression has been an independent prognostic marker for biochemical relapse and survival. (21, 54). Strikingly, different results have been reported by Bylund *et al.* (55), who found that bcl2 over-expression was associated with a better outcome and by Johnson *et al.* (56), who found bcl2 expression infrequent and without prognostic value.

Furthermore, a pro-apoptotic protein, designated as PAR4, sensitizes PC cells to apoptosis, and has been induced by growth factor starvation or serum starvation and by agents that induce apoptosis *via* the increase of intracellular Ca^{2+} in PC cells. PAR4 is expressed in both the basal and secretory cell compartment of benign prostatic tissue with nuclear and cytoplasmic localization. Moreover, the *PAR4* gene is widely expressed in different tissue types and is

exclusively induced by apoptotic stimuli. PAR4 is required, but not sufficient, to cause apoptosis in prostatic cell culture models (57). Studies in PC cell lines and fibroblasts showed that PAR4 over-expression caused a decrease in bcl2 levels and also that PAR4-dependent apoptosis is inhibited by bcl2 co-expression (58). Chakraborty *et al.* (59) reported that PAR4 drives trafficking and activation of *Fas* and *Fasl* to induce PC cell apoptosis and cancer regression, and they also suggested that PAR4 might have therapeutic potential. In human benign prostatic tissue, PAR4 expression is reported as both nuclear and cytoplasmic in the basal and secretory cell compartments, while in primary advanced cancers localization was mainly cytoplasmic and in metastatic tumors perinuclear/nuclear (58). Cook *et al.* (57) have investigated the possibility that PAR4 may be down-regulated in human cancer. They studied cases of neuroblastomas, head and neck tumors, PCs and renal cell carcinomas (RCC). PAR4 was found down-regulated only in RCCs. Because of the limited data concerning PAR4 IHC expression in PC and despite the previous negative findings, we included PAR4 in our study to analyze its possible association with bcl2 expression and the possibility of adding information for PC prognosis after RP.

Herein, certain variables known to correlate with an increasing risk for BF, such as pre-operative serum PSA levels, GS and variables of tumor pathology, such as positive surgical margins, seminal vesicle and pelvic lymph node involvement, and invasion of periprostatic tissue with bcl2, PAR4 and p53 were compared by assessing their ability to predict BF after RP. Our data showed that bcl2 over-expression does not correlate with GS or parameters related to stage of the disease, but did confirm a strong predictive value for BF after RP. The expression of p53 was correlated with invasion of the periprostatic tissue and tumor volume, showing weaker but statistically significant correlation than that of bcl2 with BF. By multivariate analysis, only infiltration of periprostatic tissue, pelvic lymph node involvement and bcl2 over-expression were documented as independent prognostic markers for BF. PAR4 was of no value for prognosis. We conclude that larger prospective studies are necessary to determine the exact prognostic value of these IHC parameters in RP specimens of clinically localized PC.

References

1 Augustin H and Hammerer PG: Disease recurrence after radical prostatectomy. Contemporary diagnostic and therapeutical strategies. Minerva Urol Nefrol *55*: 251-261, 2003.

2 Nelson JB and Lepor H: Prostate cancer: radical prostatectomy. Urol Clin North Am *30*: 703-723, 2003.

3 Apakama I, Robinson MC, Walter NM, Charlton RG, Royds JA, Fuller CE, Neal DE and Hamdy FC: bcl2 overexpression combined with p53 protein accumulation correlates with hormone-refractory prostate cancer. Br J Cancer *74*: 1258-1262, 1996.

4 Karamanolakis D, Bogdanos J, Sourla A, Milathianakis C, Tsintavis A, Lembessis P, Tenta R, Tiblalexi D and Koutsilieris M: Molecular evidence-based use of bone resorption-targeted therapy in prostate cancer patients at high risk for bone involvement. Mol Med *8*: 667-675, 2002.

5 Koutsilieris M, Lembessis P, Lieu-The V and Sourla A: Repetitive and site-specific molecular staging of prostate cancer using nested reverse transcriptase polymerase chain reaction for prostate specific antigen and prostate specific membrane antigen. Clin Exp Metastasis *17*: 823-830, 1999.

6 Mitsiades CS, Lembessis P, Sourla A, Milathianakis C, Tsintavis A and Koutsilieris M: Molecular staging by RT-PCR analysis for PSA and PSMA in peripheral blood and bone marrow samples is an independent predictor of time to biochemical failure following radical prostatectomy for clinically localized prostate cancer. Clin Exp Metastasis *21*: 495-505, 2004.

7 Thomas J, Gupta M, Grasso Y, Reddy CA, Heston WD, Zippe C, Dreicer R, Kupelian PA, Brainard J, Levin HS and Klein EA: Preoperative combined nested reverse transcriptase polymerase chain reaction for prostate-specific antigen and prostate-specific membrane antigen does not correlate with pathologic stage or biochemical failure in patients with localized prostate cancer undergoing radical prostatectomy. J Clin Oncol *20*: 3213-3218, 2002.

8 Gleason DF: Histologic grading and clinical staging of carcinoma of the prostate. *In:* Tannenbaum M (ed.), Urologic Pathology: The Prostate. Philadelphia, PA: Lea and Febiger, pp. 171-197, 1997.

9 Makarov DV, Sanderson H, Partin AW and Epstein JI: Gleason score 7 prostate cancer on needle biopsy: is the prognostic difference in Gleason scores 4+3 and 3+4 independent of the number of involved cores? J Urol *167(6)*: 2440-2442, 2002.

10 Lau WK, Blute ML, Bostwick DG, Weaver AL, Sebo TJ and Zincke H: Prognostic factors for survival of patients with pathological Gleason score 7 prostate cancer: difference in outcome between primary Gleason grades 3 and 4. J Urol *166(5)*: 1692-1697, 2001.

11 Greene FL, Page DL, Fleming ID, Fritz AG, Balch CM, Haller DG and Morrow M: AJCC Cancer Staging Manual, sixth edition, Springer, New York, Berlin, Heidelberg, Barcelona, Hong Kong, London, Milan, Paris, Singapore, Tokyo, 2002.

12 Quinn DI, Hensall SM, Head DR, Golovsky D, Wilson JD, Brenner PC, Turner JJ, Delprado W, Finlayson JF, Stricker PD, Grygiel JJ and Sutherland RL: Prognostic significance of p53 nuclear accumulation in localized prostate cancer treated with radical prostatectomy. Cancer Res *60(6)*: 1585-1594, 2000.

13 Ross JS, Jennings TA, Nazeer T, Sheehan CE, Fisher HA, Kauffman RA, Anwar S and Kallakury BV: Prognostic factors in prostate cancer. Am J Clin Pathol *120*: S85-100, 2003.

14 Pettus JA, Weight CJ, Thompson CJ, Middleton RG and Stephenson RA: Biochemical failure in men following radical retropublic prostatectomy: impact of surgical margin status and location. J Urol *172*: 129-132, 2004.

15 Kikuchi E, Scardino PT, Wheeler TM, Slawin KM and Ohori M: Is tumor volume an independent prognostic factor in clinically localized prostate cancer? J Urol *172*: 508-511, 2004.

16 Dunsmuir WD, Gillett CE, Meyer LC, Young MP, Corbishley C and Eeles RA: Molecular markers for predicting prostate cancer stage and survival. BJU Int *86*: 869-878, 2000.

17 Lane DP: P53, guardian of the genome. Nature *358*: 15-16, 1992.
18 Kastan MB, Onyekwere O, Sidransky D, Vogelstein B and Craig RW: Participation of p53 protein in the cellular response to DNA damage. Cancer Res *51*: 6304-6311, 1991.
19 Levine AJ, Momand J and Finlay CA: The p53 tumor suppressor gene. Nature *351*: 453-456, 1991.
20 Lowe SW, Schmitt EM, Smith SW, Osborne BA and Jacks T: p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature *362*: 847-849, 1993.
21 Bauer JJ, Sesterhenn IA, Mostofi FK, McLeod DG, Srivastava S and Moul JW: Elevated levels of apoptosis regulator proteins p53 and bcl2 are independent prognostic biomarkers in surgically treated clinically localized prostate cancer. J Urol *156*: 1511-1516, 1996.
22 Voeller HJ, Sugars LY, Pretlow T and Glenmann EP: *p53* oncogene mutations in human prostate cancer specimens. J Urol *151*: 492-495, 1994.
23 Navone NM, Troncoso P, Sisters LL, Goodrow TL, Palmer JL, Nichols WW, Von Eschenbach AC and Conti CJ: p53 protein accumulation and gene mutation in the progression of human prostate carcinoma. J Natl Cancer Inst *85*:1657-1669, 1993.
24 Bookstein R, MacGrogan D, Hilsenbeck SG, Sharkey F and Alfred DC: p53 is mutated in a subset of advanced-stage prostate cancers. Cancer Res *53*: 3369-3373, 1993.
25 Visacorpi T, Kallioniemi OP, Heikkinen A, Koivula T and Isola J: Small subgroup of aggressive, highly proliferative prostatic carcinomas defined by p53 accumulation. J Natl Cancer Inst *84*: 883-887, 1992.
26 Chi SG, de Vere White RW, Meyers FJ, Siders DB, Lee F and Gumerlock PH: p53 in prostate cancer: frequently expressed transition mutations. J Natl Cancer Inst *86*: 926-933, 1994.
27 Myers RB, Oelschlager D, Srivastava S and Grizzle WE: Accumulation of the p53 protein occurs more frequently in metastatic than in localized prostatic adenocarcinomas. Prostate *25*: 243-248, 1994.
28 Van Veldhuizen PJ, Sadasivan R, Cherian R, Dwyer T and Stephens RL: p53 expression in incidental prostate cancer. Am J Med Sci *305*: 275-279, 1993.
29 Tamboli P, Amin MB, Xu HJ and Linden MD: Immunohistochemical expression of retinoblastoma and p53 tumor suppressor genes in prostatic intraepithelial neoplasia: comparison of prostatic adenocarcinoma and benign prostate. Mod Pathol *11*: 247-252, 1998.
30 Haussler O, Epstein JI, Amin MB, Heitz PU and Hailemariam S: Cell proliferation, apoptosis, oncogene, and tumor suppressor gene status in adenosis with comparison to benign prostatic hyperplasia, prostatic intraepithelial neoplasia, and cancer. Hum Pathol *30*: 1077-1086, 1999.
31 Stapleton AF, Zbell P, Kattan MW, Yang G, Wheeler TM, Scardino PT and Thompson TC: Assessment of the biologic markers p53, Ki-67, and apoptotic index as predictive indicators of prostate carcinoma recurrence after surgery. Cancer *82*: 168-175, 1998.
32 Claudio PP, Zamparelli A, Garcia FU, Claudio L, Ammirati G, Farina A, Bovicelli A, Russo G, Giordano GG, McGinnis DE, Giordano A and Cardi G: Expression of cell-cycle-regulated proteins Prb2/p130, p107, p27, p53, mdm-2 and Ki-67 (MIB-1) in prostatic gland adenocarcinoma. Clin Cancer Res *8*: 1808-1815, 2002.
33 Ruijter E, van de Kaa C, Aalders T, Ruiter D, Miller G, Debruyne F and Schalken J: Heterogenous expression of E-cadherin and p53 in prostate cancer: clinical implications. BIOMED-II markers for prostate cancer study group. Mod Pathol *11*: 276-281, 1998.
34 Stackhouse GB, Sesterhenn IA, Bauer JJ, Mostofi FK, Connelly RR, Srivastava SK and Moul JW: p53 and bcl2 immunohistochemistry in pre-treatment prostate needle biopsies to predict reccurence of prostate cancer after radical prostatectomy. J Urol *162*: 2040-2045, 1999.
35 Brewster SF, Oxley JD, Trivella M, Abbott CD and Gillatt DA: Preoperative p53, bcl2, CD 44 and E-cadherin immunohistochemistry as predictors of biochemical relapse after radical prostatectomy. J Urol *161*: 1238-1243, 1999.
36 Grignon DJ, Caplan R, Sarkar FH, Lawton CA, Hammond EH, Pilepich MV, Forman JD, Mesic J, Fu KK, Abrams RA, Pajak TF, Shipley WU and Cox JD: p53 status and prognosis of locally advanced prostatic adenocarcinoma: a study based on RTOG 8610. J Natl Cancer Inst *89*: 158-165, 1997.
37 Huang A, Gandour-Edwards R, Rosenthal SA, Siders DB, Deitch AD and White RW: p53 and bcl-2 immunohistochemical alterations in prostate cancer treated with radiation therapy. Urology *51*: 346-351, 1998.
38 Matsushima H, Kitamura T, Goto T, Hosaka Y, Homma Y and Kawabe K: Combined analysis with bcl2 and p53 immunostaining predicts poorer prognosis in prostatic carcinoma. J Urol *158*: 2278-2283, 1997.
39 Oxley JD, Winkler MH, Parry K, Brewster S, Abbott C and Gillatt DA: p53 and bcl2 immunohistochemistry in preoperative biopsies as predictors of biochemical reccurence after radical prostatectomy. BJU Int *89*: 27-32, 2002.
40 Borre M, Stausbol-Gron B and Overgaard J: p53 accumulation associated with bcl2, the proliferation marker MIB-1 and survival in patients with prostate cancer subjected to watchful waiting. J Urol *164(3Pt1)*: 716-721, 2000.
41 Theodorescu D, Broder SR, Boyd JC, Mills SE and Frierson HF Jr: p53, bcl2 and retinoblastoma proteins as long-term prognostic markers in localized carcinoma of the prostate. J Urol *158*: 131-137, 1997.
42 Leibovich BC, Cheng L, Weaver AL and Bostwick DG: Outcome prediction with p53 immunostaining after radical prostatectomy in patients with locally advanced prostate cancer. J Urol *163(6)*: 1756-1760, 2000.
43 Uzoaru I, Rubenstein M, Mirochnic Y, Slobodskoy L, Shaw M and Guinan P: An evaluation of the markers p53 and Ki-67 for their predictive value in prostate cancer. J Surg Oncol *67(1)*: 33-37, 1998.
44 Van Veldhuizen PJ, Sadasivan R, Garcia F, Austenfeld MS and Stephens RL: Mutant p53 expression in prostate carcinoma. Prostate *22*: 23-30, 1993.
45 Mirchandani D, Zheng J, Miller GJ, Ghosh AK, Shibata DK, Cote RJ and Roy-Burman P: Heterogeneity in intratumor distribution of *p53* mutations in human prostate cancer. Am J Pathol *147*: 92-101, 1995.
46 Leite KR, Franco MF, Srougi M, Nesrallah A, Bevilaqua RG, Darini E, Carvalho CM, Meirelles MI, Santana I and Camara-Lopes LH: Abnormal expression of MDM2 in prostate carcinoma. Mod Pathol *14(5)*: 428-436, 2001.
47 Kallakury BV, Figge J, Ross JS, Fisher HA, Figge HL and Jennings TA: Association of p53 immunoreactivity with high Gleason tumor grade in prostatic adenocarcinoma. Hum Pathol *25*: 92-97, 1994.

48 Shiraishi T, Watanable M, Muneyuki T, Nakayama T, Morita J, Ito H, Kotake T and Yatani R: A clinicopathological study of p53, p21 (WAF1/CIP1) and cyclin D1 expression in human prostate cancers. Urol Int *61(2)*: 90-94, 1998.
49 Heidenberg HB, Sesterhenn JP, Gaddipati JP, Weghorst CM, Buzard GS, Moul JW and Srivastava S: Alteration of the tumor suppressor gene *p53* in a high fraction of hormone refractory prostate cancer. J Urol *154*: 414-421, 1995.
50 Cappello F, Rappa F, David S, Anzalone R and Zummo G: Immunohistochemical evaluation of PCNA, p53, HSP60, HSP10 and MUC-2 presence and expression in prostatic carcinogenesis. Anticancer Res *23(2B)*: 1325-1331, 2003.
51 Chao DT and Korsmeyer SJ: Bcl2 family: regulators of cell death. Annu Rev Immunol *16*: 395-419, 1998.
52 Wu TT, Hsu YS, Wang JS, Lee YH and Huang JK: The role of p53, bcl2 and E-cadherin expression in predicting biochemical relapse for organ confined prostate cancer in Taiwan. J Urol *170(1)*: 78-81, 2003.
53 McDonell TJ, Troncoso P, Brisbay SM, Logothetis C, Chung LW, Hsieh JT, Tu SM and Campbell ML: Expression of the proto-oncogene *bcl2* in the prostate and its association with emergence of androgen-independent prostate cancer. Cancer Res *52*: 6940-6944, 1992.
54 Moul JW, Bettencourt MC, Sesterhenn IA, Mostofi FK, McLeod DG, Srivastava S and Bauer JJ: Protein expression of p53, bcl2 and Ki-67 (MIB-1) as prognostic biomarkers in patients with surgically treated, clinically localized prostate cancer. Surgery *120*: 159-166, 1996.
55 Bylund A, Stattin P, Widmark A and Bergh A: Predictive value of bcl2 immunoreactivity in prostate cancer patients treated with radiotherapy. Radiother Oncol *49*: 143-148, 1998.
56 Johnson MI, Robinson MC, Marsh C, Robson CN, Neal DE and Hamdy FC: Expression of bcl2, bax, and p53 in high-grade prostatic intraepithelial neoplasia and localized prostate cancer: relationship with apoptosis and proliferation. Prostate *37*: 223-229, 1998.
57 Cook J, Krishan S, Anath S, Sells SF, Shi Y, Walther MM, Linehan WM, Sukhatme VP, Weinstein MH and Ragnekar VM: Decreased expression of the pro-apoptotic protein PAR4 in renal cell carcinoma. Oncogene *18*: 1205-1208, 1999.
58 Qiu G, Ahmed M, Sells SF, Mohiuddin M, Weinstein M and Ragnekar VM: Mutually exclusive expression patterns of bcl2 and PAR4 in human prostate tumors consistent with down-regulation of bcl2 by PAR4. Oncogene *18*: 623-631, 1999.
59 Chacraborty M, Qiu SG, Vasudevan KM and Rangnekar VM: PAR4 drives trafficking and activation of Fas and Fasl to induce prostate cancer cell apoptosis and tumor regression. Cancer Res *61(19)*: 7255-7263, 2001.

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