Oncology Research, Vol. 12, pp. 43–49, 2000
Printed in the USA.

0965-0407/00 $20.00 + .00

Expression of p53 and bcl-2 in Clinically Localized Prostate Cancer Before and After Neo-Adjuvant Hormonal Therapy

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(Submitted September 9, 1999; sent for revision November 1; received April 19, 2000; accepted April 20, 2000)

The prognostic significance of p53 and bcl-2 expression in prostate carcinoma is currently under investigation. The aim of the present study was to analyze their expression in diagnostic biopsies and in prostatectomies performed after neo-adjuvant hormonal therapy to investigate their role in hormone resistance. One hundred and six patients with advanced prostate carcinoma were treated for 3 months with LHRH analogues before radical surgery. The expression of p53 and bcl-2 was analyzed by immunohistochemistry in all cases of prostatectomy and in available biopsies obtained before treatment, and was correlated with clinicopathologic parameters and follow-up. A significant increase in p53 expression was found following hormonal therapy, whereas no changes were observed in the expression of bcl-2. The increase in p53 did not correlate with the presence of therapy-induced morphological changes in prostate cancers, but it did correlate significantly with histologic grade and pathologic stage, biochemical progression of the disease, and short overall survival. At multivariate analysis, only grade and stage proved to be independent predictors of shorter survival. There were no correlations between bcl-2 and clinicopathologic variables whether in biopsies or in prostatectomies. The unfavorable clinical course associated with p53-positive carcinomas suggests that neo-adjuvant hormonal therapy may cause the selection of minor p53 mutated clones, rather than the induction of wild-type p53. In any case, the enhanced expression of p53 could label hormone-resistant cancers for further adjuvant therapy.

Key words: Prostate cancer; Hormonal therapy; p53; bcl-2; Hormone resistance; Prognosis

Cancer of the prostate is one of the most frequent malignancies in human males, and several studies have been undertaken to clarify its biological behavior, particularly in relation to recent therapeutic advances. In particular, the molecular mechanisms responsible for tumor progression and androgen resistance are being actively studied.

The role of genes such as bcl-2 and p53 in carcinogenesis has been widely investigated but is still a matter of debate. Mutation of the tumor suppressor gene Tp53 has been documented in a large number of human malignancies (1), whose prognosis therefore seems to be negatively influenced (2–4). In prostate carcinoma, a more powerful expression has been observed in high-grade tumors (5–7), as well as a significant correlation with stage, thus emphasizing the role of the gene in the process of tumor progression (5–8). Bcl-2 overexpression is associated with reduced apoptosis and extension of cell life span, which allows genetic alterations to accumulate (9). In the adult prostate it has been suggested that bcl-2 is inversely related to androgen stimulation, normally being expressed in the basal cell layer of glands but lacking in the luminal cells. Expression of bcl-2 has been found in prostate cancer (10,11), in prostatic intraepithelial neoplasia (PIN[2]), and in hormone-refractory cancers (12). These findings suggest that bcl-2 may initiate carcinogenesis, as well as conferring resistance on prostatic tumor cells in an androgen-deprived environment (12).

The efficacy of hormonal therapy with luteinizing hormone-releasing hormone (LHRH) analogues in the treatment of androgen-dependent prostatic carcinomas is well known. Nevertheless, it is not clear whether the growth inhibition and apoptosis induced by therapy are related to changes in the expression of the proteins involved in the apoptotic pathway. Recent experimental studies (13,14) did not demonstrate increased p53 expression after androgen withdrawal, nor was any prognostic value of bcl-2 expression found in some cases treated with hormonal therapy (15). Similarly, no significant posttreatment differences in bcl-2 expression were observed between nonrelapsed and relapsed tumors (16).

Conversely, an increased expression of p53 was detected in a high fraction of spontaneous prostate cancer hormone refractory, but any possible correlation with overall survival was not searched for (17–19).

In the present study, conducted on a group of patients with clinically localized prostate cancer, we evaluated p53 and bcl-2 protein expression in needle biopsies and in corresponding surgical specimens removed from patients undergoing radical prostatectomy after hormone treatment. Our aim was to find out whether different

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[2]Abbreviations used: PIN, prostatic intraepithelial neoplasia; LHRH, luteinizing hormone-releasing hormone; PSA, prostatic-specific antigen.

gene expression could be observed after hormone treatment in these relatively precocious tumors and, if so, to consider the possible relationship between the expression of the genes and the mechanisms of response to such therapy.

MATERIALS AND METHODS

Over a period of approximately 7 years (1990–1996), 106 consecutive patients with a prostate cancer apparently limited to the gland at clinical examination were diagnosed at needle biopsy as having prostate carcinoma. Patients who refused preoperative hormonal therapy and patients lost at follow-up were excluded from the study. The mean age of the patients was 66 years (range 50–72). The serum level of prostatic-specific antigen (PSA) was available in all patients but three; it was less than 4.0 ng/ml in 11 patients, between 4.1 and 10 ng/ml in 21 patients, and more than 10 ng/ml in the other 71. All patients were similarly treated for 3 months with the same LHRH analogues before undergoing radical surgery. The grade of the tumor, established according to Gleason's method (20) on the diagnostic core biopsy to avoid possible modifications induced by therapy, was 2–4 in 30 cases, 5–7 in 67 cases, and 8–10 in 9 cases.

Immunohistochemistry

The immunohistochemical detection of p53 and bcl-2 proteins was performed on 4-μm sections from routinely processed, formalin-fixed, paraffin-embedded tissue blocks employing an avidin-biotin complex immunoperoxidase technique. In the case of radical prostatectomy, a single block with the largest focus of cancer was selected for each case. In the case of biopsy, residual material available for immunohistochemistry was present only in 77 cases. For antigen retrieval, the slides were pretreated in citrate buffer at pH 6 in a microwave oven for 30 min at 360°C. Only in the case of surgical specimens was p53 staining performed even without microwave pretreatment. Anti-p53 monoclonal antibody DO-7 (Dako, Glostrup, Denmark) was diluted 1:800 and incubated overnight at 4°C. Monoclonal antibody anti-bcl-2 (clone 124 IgG1) (YLEM, Rome, Italy) was diluted 1:40 and incubated at room temperature for 1 h. The ABC method (Vector Laboratories, Burlingame, CA) with diaminobenzidine was used as detection system. A p53-positive colonic adenocarcinoma and a normal tonsil were used as positive controls for p53 and bcl-2, respectively. An internal control for bcl-2 was also represented by lymphocytes and basal cells of normal glands present in the section. Slides incubated without primary antibody were used as negative controls. The results were evaluated by calculating the percentage of neoplastic cells stained positively by primary antibody out of a total of 1000 cells in surgical specimens and at least 200 cells in bioptic samples. Nuclear and cytoplasmic staining was considered for p53 and bcl-2, respectively, regardless of the staining intensity of the reaction. Results of p53 staining were scored as follows: 0 = completely negative; 1, 2, and 3 = <10%, 10–30%, and >30% positive, respectively. Immunostaining for bcl-2 was scored as: 0 = completely negative; 1, 2, and 3 = <10%, 10–50%, and >50% positive, respectively.

Statistical Analysis

The chi-square test was used to define the statistical differences of the variables analyzed. Survival curves were calculated using the Kaplan-Meier method and the difference between curves was tested by the log-rank test. Multivariate analysis was performed by Cox regression analysis. The analysis of disease-free survival was not possible, because the time of biochemical progression after surgery was not assessed. Results were considered statistically significant if $P \leq 0.05$.

RESULTS

After a median follow-up of 49 months (range 20–90 months), 46 patients were alive and free of disease, while 60 showed biochemical progression, as assessed by two successive serum measurements of PSA both higher than 0.3 ng/ml (Tandem assay, Hybritech Incorporated, San Diego, CA). In the first group, one patient died of other causes during follow-up, whereas 13 patients in the group displaying biochemical progression died from tumor progression. At surgery, the histopathologic examination of the prostatectomy specimens showed that 58 patients were at the pT2N0 stage and 31 at the pT3N0 stage, while 17 presented lymph node metastases. Morphological changes induced by hormonal therapy, similar to those described by Murphy et al. (21), were found in 56 out of 106 cases (52.8%). In 50 cases not showing any therapy-induced morphological changes there was no substantial change in the Gleason grade, as reassessed in surgical specimens, with respect to that assessed at preoperative biopsy. In the remaining 56 cases, the presence of therapy-induced morphological changes made the evaluation of Gleason grade unreliable, as pointed out in several previous studies (21–23).

At immunohistochemical analysis performed without antigen retrieval, p53 nuclear positivity was observed in 32 out of 106 radical prostatectomies (30.2%) and the score was above 1 in 12 cases (11.3%). When immunohistochemistry was performed with antigen retrieval, p53 was positive in 77 cases (72.6%) and the score was greater than 1 in 37.7% of the cases (29 scored 2 and 11 scored 3). No nuclear staining was found in nonneoplastic tissue; a weak cytoplasmic positivity was observed only in urothelial cells lining the prostatic urethra. On the other hand, the expression of p53 in preoperative biopsies was significantly lower than that observed in surgical specimens ($p < 0.0001$); in fact, even after antigen retrieval, there were only 8 positive cases out of the 77 tested (10.67%) and the score was 1 in all cases. No significant correlations were found when the expression of p53 in preoperative biopsies was compared with that in prostatectomies; in particular, 8 cases were positive and 21 negative both in bioptic and in surgical specimens, while 48 cases negative at biopsy were positive at prostatectomy.

The expression of bcl-2 was successfully analyzed in 102 surgical specimens; in spite of repeated staining attempts, no adequate reaction was achieved in the last 4 cases, as proven by the negative internal controls. In all the other cases, the internal controls were positive, as were nonneoplastic epithelial cells exhibiting features of urothelial and squamous metaplasia. Positive cancer cells were found in 11 cases (10.5%), the score being 1 in 6 cases, 2 in 1 case, and 3 in 4 cases. Similar results were obtained from the 71 cases studied at biopsy, 9 of which were positive (12.67%), with a score of 1 in 4 cases, 2 in 2 cases, and 3 in the last 3 cases. Also, no correlation was found between the expression of bcl-2 in biopsies and that in prostatectomies; in particular, 4 cases were positive in both samples, 4 cases were positive only in bioptic, and 3 only in surgical specimens. However, it is noteworthy that the three cases with a score of 3 at biopsy presented the same high score at prostatectomy.

Follow-up appeared to correlate strongly with the Gleason grade ($P = 0.003$) and pathologic stage ($P = 0.001$), while there were no correlations with age, therapy-induced changes, and bcl-2 expression. The Gleason grade did not correlate with stage ($P = 0.2$) (Table 1). Preoperative PSA level did not show any correlation with grade, stage, or follow-up, whether with p53 or bcl-2 expression in bioptic material. Conversely, the rise in PSA level after surgery was followed by death due to tumor progression in 13 out of 60 patients (21.66%). Similarly, no correlations were found between p53 expression at biopsy and tumor grade, pathologic stage, and follow-up (data not shown). On the other hand, p53 immunostaining after microwave pretreatment in surgical specimens appeared to correlate with tumor grade, pathologic stage, and follow-up, findings mainly due to cases which scored 3 (Table 2). The expression of p53 did not correlate with that of bcl-2 in either bioptic or surgical specimens. In particular, of the biopsies, 2 were positive for both proteins, 6 only for bcl-2, and 5 only for p53; in the surgical specimens, 8 of the 11 bcl-2-positive cases were also positive for p53. Bcl-2 expression did not show any significant correlation with pathologic parameters, whether at prostatectomy or biopsy (data not shown). When the effect of therapy (i.e., the presence of therapy-induced morphological changes) was evaluated in relation to p53 and bcl-2 expression, pathologic grade and stage, and follow-up, no significant correlations were found.

Overall survival appeared to be influenced by the Gleason grade (log-rank test 6.15, $P = 0.046$), pathologic stage (log-rank test 7.79, $P = 0.020$), and biochemical progression (log-rank test 5.43, $P = 0.019$), but not by age (log-rank test 0.62, $P = 0.431$), therapy-induced changes (log-rank test 1.09, $P = 2.95$), or oncoprotein expression in preoperative biopsy. On the other hand, the expression of p53 in surgical specimens appeared to correlate strongly with shorter survival (log-rank test 11.44, $P = 0.009$). This was true, in particular, for cases with a percentage of p53-positive cells higher than 30%, while lower scores of positivity did not appear to differ from negative cases (Fig. 1). No correlation was found between overall survival and bcl-2 expression in surgical specimens (log-rank test 0.01, $P = 0.919$). When a Cox proportional-hazard model was constructed that included the age of patients at the time of diagnosis, therapy-induced changes, tumor grade and stage, and p53 and bcl-

Table 1. Follow-Up and Clinicopathological Data in 105 Primary Prostatic Carcinomas*

		Follow-Up			
	Case	Alive, Disease Free	Biochemical Progression	Died of Disease	*P*-Value
Age (years)					0.925
≤60	21	9	10	2	
>60	84	36	37	11	
Tumor grade					0.003
2–4	30	18	11	1	
5–7	67	26	33	8	
8–10	8	1	3	4	
Tumor stage					0.001
pT_2N0	57	34	20	3	
pT_3N0	31	11	17	3	
$pT_{2\text{-}3}N+$	17	0	10	7	
Therapy-induced changes					0.812
Yes	55	25	24	6	
No	50	20	23	7	
Bcl-2 expression†					0.796
Positive	11	4	5	2	
Negative	91	40	41	10	

*One patient excluded (death due to other causes).
†102 cases out of 106 with available reaction.

Table 2. p53 Expression in Prostatectomy Specimens and Clinicopathological Data in 106 Primary Prostatic Carcinomas

		p53 Staining Score				
	Case	Neg.	<10	10–30	>30	*P*-Value
Age (years)						0.570
≤60	21	4	10	5	2	
>60	85	25	27	24	9	
Tumor grade						0.018
2–4	30	3	16	9	2	
5–7	67	24	18	19	6	
8–10	9	2	3	1	3	
Tumor stage						0.040
pT_2N0	58	23	17	15	3	
pT_3N0	31	4	14	9	4	
$pT_{2\text{-}3}N+$	17	2	6	5	4	
Therapy-induced changes						0.422
Yes	56	17	22	12	5	
No	50	12	15	17	6	
Bcl-2 expression*						0.649
Positive	11	3	4	4	0	
Negative	91	26	33	25	11	
Follow-up						0.004
Alive, disease free	45	17	17	8	3	
Biochemical progression	47	11	15	18	3	
Died of disease†	13	1	4	3	5	

*102 cases out of 106 with available reaction.
†One patient excluded (death due to other causes).

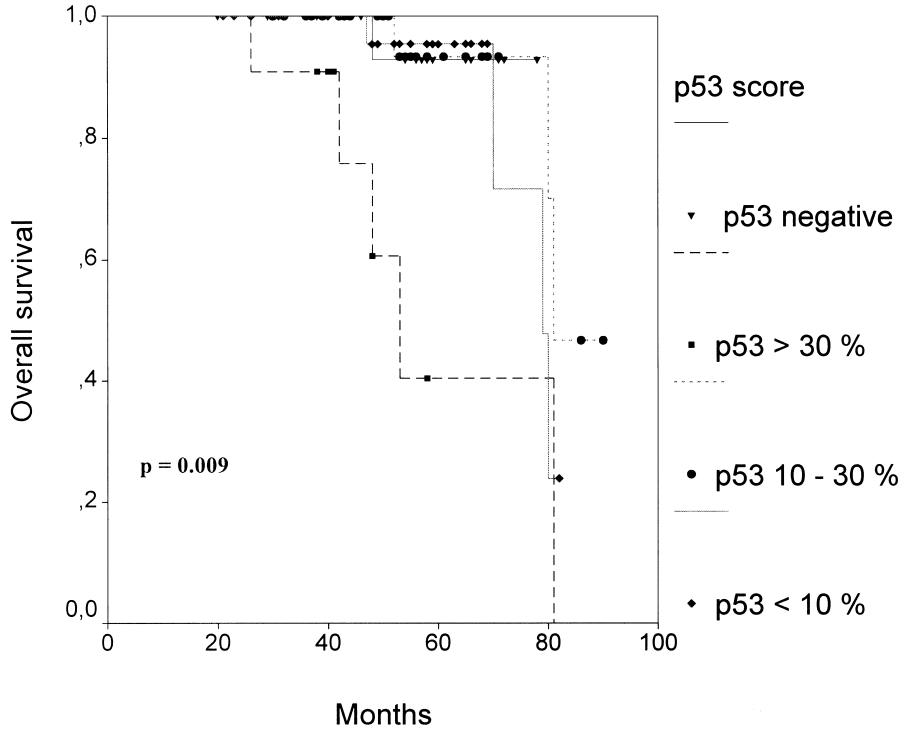
2 expression, grade and stage were the only independent predictors of reduced overall survival ($P = 0.001$ and $P = 0.02$, respectively) (Table 3).

DISCUSSION

The increase in prostate carcinoma, particularly in recent years, has aroused great interest. Several studies have been carried out in order to identify reliable parameters, other than the classic Gleason grade and pathologic stage, to predict its biological behavior. Hormonal therapy, as an alternative or adjunct to surgery in the treatment of prostate cancer, has been reported to increase the programmed death of tumor cells (24), thus improving patients' survival, but androgen resistance has been observed to develop in many cases (25).

The role of p53 and bcl-2 oncoproteins in apoptotic mechanisms has been widely researched (5,8,10–12,26,27). In the present study, using an immunohistochemical approach, we investigated the changes in the expression of p53 and bcl-2 in prostate cancer occurring after neo-adjuvant therapy with LHRH analogues and correlated the results with follow-up and overall survival. While no changes were observed in bcl-2 expression, a significant increase was detected in the expression of p53, which became positive in 72.6% of the cases and correlated with shorter survival.

The value of p53 in the prognostic evaluation of prostate cancer has been pointed out in many studies (5,8,26–31), but overly variable degrees of positivity have been reported, ranging from 12% (32) to 79% (33). As often stressed (19,34), the discrepancy in immunohistochemical results is very common and may be due to various factors, such as the use of different antibodies, different antigen unmasking procedures, and, particularly, the different cutoffs adopted to define p53 positivity. In fact, in the present study, the expression of p53, evaluated either with or without microwave pretreatment, was found to be present in significantly higher percentages of tumor cells after microwave heating, and significant correlations with Gleason grade, pathologic stage, and follow-up were observed only after this procedure. In keeping with Berner et al. (18) and Koivisto and Rantala (19), who found p53 overexpression in about 40% of hormonally treated, locally recurrent prostate cancers, our rate of positivity decreased from 72.6% to 37.7% when cases with more than 10% positive cells were considered. On the other hand, the expression of p53 in preoperative needle biopsy, even after antigen retrieval, was significantly lower than that detected in surgical specimens, and did not show any significant correlation with tumor grade, stage, and follow-up. Accordingly, the expression of p53 in biopsy is a matter for debate, because some studies have found significant



p53 score	Total cases	Number of events	Overall survival (months)
p53 < 10 %	36	4	76
p53 10 – 30 %	29	3	83
p53 > 30 %	11	5	59
p53 negative	29	1	76
Total	105	13	

Figure 1. Kaplan-Meier curve for overall survival in 105 patients who underwent neo-adjuvant hormonal therapy and radical surgery for primary prostate carcinoma stratified according to p53 expression. Increased p53 expression >30% was significantly associated with overall survival (P = 0.009 by log-rank test).

Table 3. Contribution of Various Potential Prognostic Factors to Overall Survival by Cox Regression Analysis in 105 Primary Prostate Carcinomas

	Variables in the Equation						
Variable	B	SE	Wald	*df*	Sig.	*R*	exp(B)
Age	1.4999	0.8769	2.9257	1	0.0872	0.1068	4.4811
Therapy	0.9015	0.6984	1.6661	1	0.1968	0.0000	0.4060
Gleason	1.8129	0.5552	10.6637	1	0.0011	0.3267	6.1281
Stage	1.1443	0.5030	5.1752	1	0.0229	0.1978	3.1401
p53	0.1291	0.3167	0.1662	1	0.6835	0.0000	1.1378
BCL-2	1.1192	0.8800	1.6177	1	0.2034	0.0000	3.0626

correlation with a worse clinical outcome (29–31), while others have excluded any significant correlation (19,35,36), on the grounds of the heterogeneous distribution of p53 (37).

On the other hand, bcl-2 was expressed in a low percentage of cases, both in biopsy and in prostatectomy. Unlike some previous studies, which showed significant correlation between bcl-2 expression and prostate cancer aggressive behavior (10,11,38) or androgen independency (12,39), the present work found no such correlation. This is in agreement with other studies, in which bcl-2 expression did not show prognostic relevance either in prostatectomy (28) or in biopsy (29–31,35,36). Moreover, the level of bcl-2 expression did not appear to change significantly after therapy, nor was any change in level observed between cases with or without morphological alterations induced by treatment. Westin et al. (40) and Stattin et al. (16) found increased bcl-2 expression in biopsies obtained after castration therapy in both responding and nonresponding patients, but, as in the present study, immunoreactivity for bcl-2 did not vary according to subsequent clinical response or to the presence of therapy-induced morphological changes. In the study of Noordzij et al. (15), bcl-2 expression was not significantly modified by therapy in androgen-independent tumors, nor did it show prognostic significance in tumors before treatment, or correlate with pathologic stage or histologic grade. In support of this view, Woolveridge et al. (41) showed that bcl-2 family members do not seem to be important in the apoptosis that characterizes rat ventral prostate regression following androgen withdrawal.

Koivisto et al. (25) suggested that many mechanisms are involved in the failure of hormonal therapy. In our experience, the expression of bcl-2 did not seem to be influenced by androgen ablation, nor to affect the clinical course of the disease after hormonal therapy. On the other hand, p53 expression was significantly increased in tumors after hormonal treatment, as previously shown by Heidenberg et al. (17), Berner et al. (18), and Koivisto and Rantala (19), and appeared to select the patients prone to progression and with a significantly shorter survival. These results were unrelated to the morphological changes induced by therapy and suggested that the difference in p53 expression between preoperative biopsies and prostatectomies is unlikely to be merely due to the small number of neoplastic cells present in the former and to the low percentage (<10%) of positive cells, in about half of positive surgical specimens. Moreover, if tumor heterogeneity and differences in cell sampling were the reason for the difference in p53 expression, there should be an analogous difference in bcl-2 expression between biopsy and prostatectomy. An increase in p53 expression following castration therapy was observed by Stattin et al. (16) in responding prostate cancer patients and it was interpreted as being due to the induction of wild-type p53. The lack of significant change in the level of p53 expression after androgen ablation led Furuja et al. (13) and Woolveridge et al. (41) to state that hormonal-induced apoptosis of prostatic glandular cells was a p53-independent process. In our study, enhanced p53 expression was found in surgical specimens, but with no significant difference between cases with or without therapy-induced morphological changes. Moreover, the protein expression appeared to correlate with advanced pathologic stage, disease progression, and unfavorable clinical outcome.

As suggested by Lowe et al. (42) in the case of chemotherapy, it is possible that androgen ablation may cause the selection of minor Tp53-mutated clones, which subsequently proliferate, providing the genetic basis for hormone resistance and for prostate cancer progression (19). However, the lack of an independent prognostic value of p53 expression argues against such possible role. Only the molecular assessment of Tp53 gene status in tumors with increased p53 expression and short survival after neo-adjuvant hormonal therapy would be useful to confirm this hypothesis and help in selecting patients for further adjuvant treatment.

ACKNOWLEDGMENTS: The authors thank Romana Iotti, Paola Manni, and Elisabetta Mattioli for their skillful technical assistance in carrying out immunohistochemical assays. The work was supported in part by a grant from cofinanziamento *MURST 1998 and by a grant from the University of Modena and Reggio Emilia.*

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