ACCUMULATION OF NUCLEAR p53 AND TUMOR PROGRESSION IN BLADDER CANCER

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Abstract *Background.* We have previously demonstrated a strong association between nuclear accumulation of p53 protein, as determined by immunohistochemical analysis, and mutations in the p53 gene. The purpose of this study was to determine the relation between nuclear accumulation of p53 and tumor progression in transitional-cell carcinoma of the bladder.

Methods. Histologic specimens of transitional-cell carcinoma of the bladder (stages Pa, noninvasive disease, to P4, disease with direct extension into adjacent organs or structures) from 243 patients who were treated by radical cystectomy were examined for the immunohistochemical detection of p53 protein. Nuclear p53 reactivity was then analyzed in relation to time to recurrence and overall survival.

Results. The detection of nuclear p53 was significantly associated with an increased risk of recurrence of bladder cancer ($P<0.001$) and with decreased overall survival ($P<0.001$). In patients with cancer confined to the bladder, the rates of recurrence for stage P1, P2, and P3a tumors that had no detectable nuclear p53 reactivity at five years were 7, 12, and 11 percent, respectively, as compared with 62, 56, and 80 percent, respectively, for tumors that had p53 immunoreactivity. Similar results were obtained when the presence or absence of p53 in the nuclei of the tumor cells was studied in relation to overall survival. In a multivariable analysis stratified according to grade, pathological stage, and lymph-node status, nuclear p53 status was an independent predictor (and in cancer confined to the bladder, the only independent predictor) of recurrence and overall survival ($P<0.001$).

Conclusions. In patients with transitional-cell carcinoma confined to the bladder, an accumulation of p53 in the tumor-cell nuclei detected by immunohistochemical methods predicts a significantly increased risk of recurrence and death, independently of tumor grade, stage, and lymph-node status. Patients with transitional-cell carcinoma confined to the bladder that demonstrates nuclear p53 reactivity should be considered for protocols of adjuvant treatment. (N Engl J Med 1994;331:1259-64.)

MUTATIONS of the p53 gene are the most common genetic defect in human tumors.[1] The p53 gene functions as a tumor-suppressor gene and more specifically as a cell-cycle regulator.[2] Levels of p53 protein increase in response to damage to DNA, arresting the cell cycle and allowing time for the repair of DNA.

Mutations of the p53 gene occur in a high percentage of invasive transitional-cell carcinomas of the bladder[3] and appear to be an early event in the formation of carcinoma in situ.[4] They are much less frequent in noninvasive papillary tumors.[5,6] Mutations of the p53 gene and nuclear accumulation of p53 protein are associated with the grade and stage of bladder cancer[5] and may be important in the multistep progression of bladder cancer.[7-9] The immunohistochemical detection of the protein, which exploits the difference in life span between mutated and wild-type p53, has been shown to correlate strongly with mutations of the p53 gene in bladder cancer.[5]

The primary factors used in determining the clinical treatment of patients with early-stage bladder cancer are the depth of tumor invasion, the tumor grade, and the presence or absence of lymph-node metastases. Radical cystectomy is a generally accepted treatment for patients with invasive cancer confined to the bladder, particularly when there is evidence of muscle invasion.[10] Surgical treatment is curative in a substantial proportion of such patients, but in a large number incurable metastatic disease appears after surgery. Adjuvant therapy (including chemotherapy and radiation therapy) is under investigation in this setting, and shows encouraging results.[11,12] It may thus be important to distinguish patients for whom surgery alone is adequate and who can avoid the morbidity and expense of adjuvant therapy from patients who might benefit from both surgery and additional treatment.

In the present study we sought to determine whether a nuclear accumulation of p53 is an important factor in predicting the clinical behavior of bladder cancer. Previous work has suggested that alterations of p53 can predict disease progression in superficially invasive bladder cancer.[13] However, a study of patients with bladder cancer who received a variety of treatments suggested that nuclear accumulation of p53 is not a predictor of disease progression that is independent of clinical stage and the rate of proliferation of the tumor cells.[14] We demonstrate, in 243 patients with bladder cancer treated by radical cystectomy, that nuclear accumulation of p53 identifies transitional-cell carcinomas with a propensity for progression that is independent of tumor grade and stage.

METHODS

Population of Patients

We studied 243 patients who underwent radical cystectomy, pelvic-lymph-node dissection, and urinary diversion at the Kenneth Norris Jr. Comprehensive Cancer Center. To assess the association between nuclear accumulation of p53 and clinical outcome, we included all patients with transitional-cell carcinoma treated by radical cystectomy from April 1983 through June 1987 for whom follow-up data and tumor samples from the cystectomy specimens

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(preserved in archival paraffin-embedded tissue blocks) were available. An additional 33 patients who underwent cystectomy between July 1987 and December 1988 were studied in order to increase the number of patients with cancer confined to the bladder. The study also included 53 patients described previously whose tumors underwent both molecular and immunohistochemical analysis for p53.[5] Thus, of the entire group of 243 patients, 190 had not previously been evaluated for nuclear accumulation of p53. Patients with pure adenocarcinoma, squamous-cell carcinoma, or small-cell carcinoma of the bladder were excluded. The median age of all patients was 63 years (range, 49 to 83); 77 percent were men, and 23 percent were women.

Clinical and Pathological Evaluation

The indications for radical cystectomy included invasion of muscle or prostate stroma by the tumor; high-grade, superficially invasive tumors associated with carcinoma in situ; carcinoma in situ refractory to intravesical chemotherapy or immunotherapy; and recurrent multifocal disease after conservative therapy. None of the patients received pelvic irradiation or systemic chemotherapy before surgery. All the specimens included in this study were transitional-cell carcinomas; a minority demonstrated glandular or squamous differentiation. The histologic grading was performed according to the method of Bergkvist et al.,[15] and the pathological staging was done according to the tumor–node–metastasis classification.[16] All tumors were reevaluated for histologic grade by two of the investigators. Among the 243 tumors, 11 were classified histologically as grade 2, 157 as grade 3, and 75 as grade 4. Five tumors were classified as stage Pa (noninvasive papillary tumors), 11 as stage Pis (carcinoma in situ), 50 as stage P1 (superficial invasion), 32 as stage P2 (superficial invasion into muscularis propria), 35 as stage P3a (deep invasion into muscularis propria), 68 as stage P3b (invasion into perivesicular fat), and 42 as stage P4 (direct extension into adjacent organs or structures). Sixty-six patients were found on pathological examination to have metastatic disease in the pelvic lymph nodes. None of the patients had distant metastatic disease at the time of cystectomy. Forty-one patients received systemic chemotherapy after surgery, either cisplatin, cyclophosphamide, and doxorubicin or methotrexate, vinblastine, doxorubicin, and cisplatin. The median follow-up for the 243 patients was 6.0 years, with 81 percent having at least 3 years of follow-up. The patients were seen at three-month intervals during the first postoperative year, every four months during the second year, and every year thereafter. Follow-up consisted of a biochemical profile, chest radiography, and physical examination. A computed tomographic scan or bone scan was performed to confirm suspected recurrences of disease. Data on recurrences of transitional-cell carcinoma and causes of death were obtained from office and hospital records.

Monoclonal Antibodies and Immunohistochemical Analysis

Five-micrometer sections of archival formalin-fixed, paraffin-embedded tissue were placed on slides coated with poly-L-lysine (Sigma, St. Louis). The immunohistochemical procedure was performed as described elsewhere.[5] In brief, after deparaffinization and blocking of endogenous peroxidase, the anti-p53 mouse monoclonal

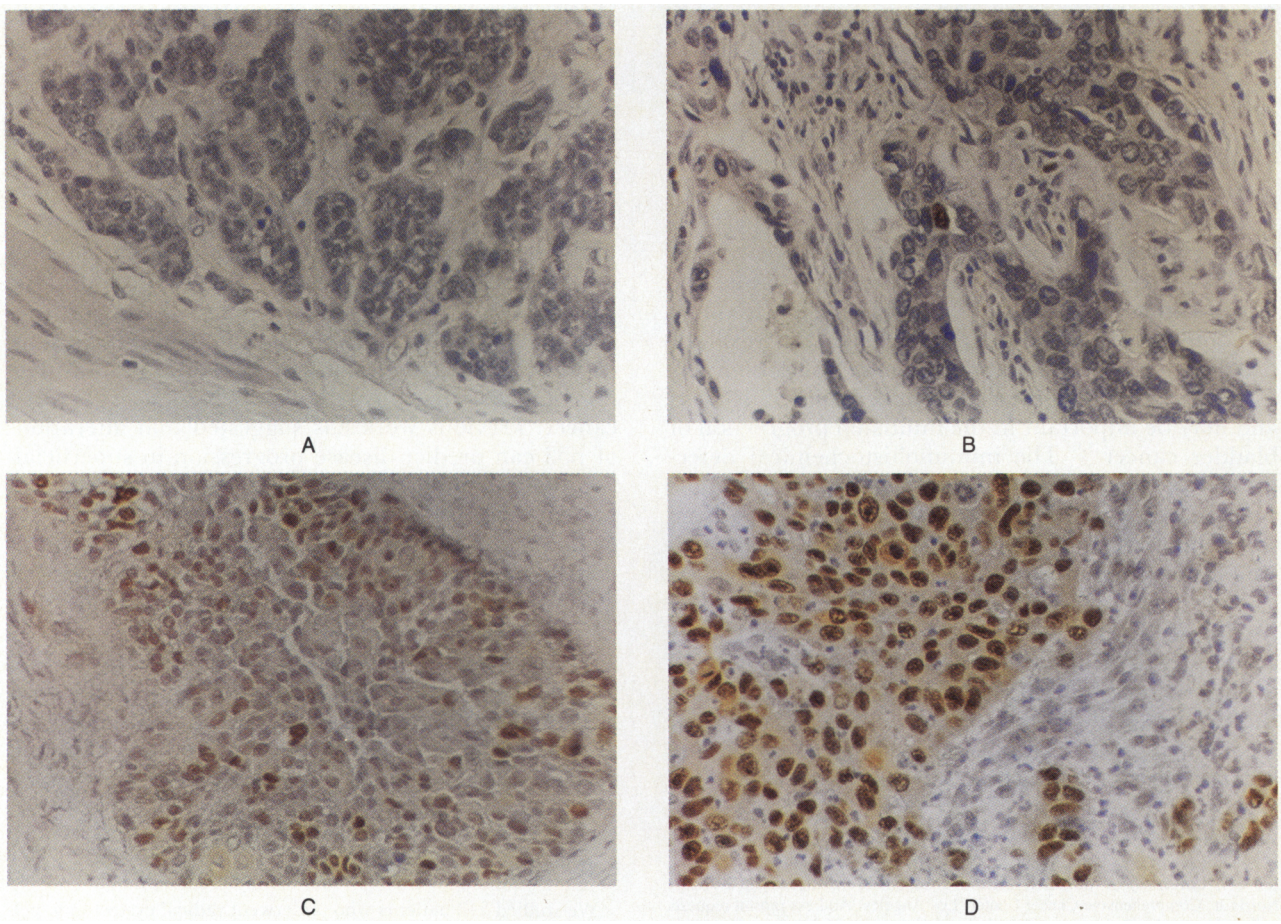

Figure 1. Immunohistochemical Detection of p53 Protein in the Nuclei of Transitional-Cell Carcinomas of the Bladder with the Anti-p53 Monoclonal Antibody PAb1801.

No detectable nuclear staining is seen in Panel A, only a few cells (1 to 9 percent) with nuclear reactivity are seen in Panel B, heterogeneous nuclear reactivity is seen in 10 to 49 percent of tumor cells in Panel C, and intense homogeneous nuclear reactivity is seen in 50 to 100 percent of tumor cells in Panel D (all panels ×105). Tumors with 0 to 9 percent nuclear staining were considered p53-negative, whereas tumors with 10 to 100 percent nuclear staining were considered p53-positive.

Table 1. Association of p53 Immunoreactivity with Grade and Pathological Stage of Bladder Cancer and Presence or Absence of Lymph-Node Metastases in 190 Patients Not Previously Tested for p53 Alterations.

VARIABLE	NO. OF PATIENTS	NUCLEAR p53 REACTIVITY*		P VALUE
		NEGATIVE	POSITIVE	
		number (percent)		
Grade†				
2	9	7 (78)	2 (22)	
3	122	79 (65)	43 (35)	
4	59	33 (56)	26 (44)	0.20‡
Stage§				
Metastases absent				
Pa or Pis	15	12 (80)	3 (20)	
P1	39	30 (77)	9 (23)	
P2	29	19 (66)	10 (34)	
P3a	20	11 (55)	9 (45)	
P3b	36	21 (58)	15 (42)	
P4	9	7 (78)	2 (22)	
Metastases present	42	19 (45)	23 (55)	0.003¶

*Negative indicates that less than 10 percent of tumor-cell nuclei demonstrated p53 immunoreactivity, and positive that 10 percent or more of tumor-cell nuclei demonstrated p53 immunoreactivity.

†According to the method of Bergkvist.[15]

‡Derived from Pearson's chi-square test for a two-by-two table comparing grade 2 and 3 tumors with grade 4 tumors.[17]

§Pathological staging was performed according to the tumor–node–metastasis system.[16] Metastases refers to lymph-node metastases identified during the pathological evaluation.

¶Based on the likelihood-ratio test for logistic regression.[17]

antibody PAb1801 (IgG1 class, 1:10 dilution, Biogenex, San Ramon, Calif.) was incubated overnight with tissue at 4°C. PAb1801 recognizes the p53 protein at a denaturation-resistant epitope corresponding to amino acids 32 through 79. Tissues were then incubated with a biotinylated horse antimouse secondary antibody (Vector Labs, Burlingame, Calif.), and reactivity was visualized with an avidin–biotin–immunoperoxidase system (Vector) using diaminobenzidine (0.03 percent) as the chromogen and hematoxylin as the counterstain. Samples of bladder carcinoma with known p53 mutations and documented accumulations of p53 protein by immunohistochemical analysis were used as positive controls. Normal urothelium and nonepithelial cells (lymphocytes, stromal cells, and endothelial cells), used as internal negative controls, demonstrated no immunoreactivity. Only nuclear localization of immunoreactivity was evaluated. The extent of nuclear reactivity was classified in four categories: no nuclear reactivity; a few focally positive nuclei (1 to 9 percent of tumor cells); heterogeneous nuclear reactivity (10 to 49 percent of tumor cells); and intense homogeneous nuclear reactivity (50 to 100 percent of tumor cells) (Fig. 1). Only samples demonstrating at least 10 percent nuclear reactivity were considered to be p53-positive (to have an alteration in p53). We based this criterion on our demonstration of a strong correlation of mutations in the p53 gene with the accumulation of p53 protein in 10 percent or more of the tumor-cell nuclei.[5] The immunohistochemical analysis was performed without knowledge of the tumor stage or the results of clinical follow-up.

Statistical Analysis

Survival was calculated from cystectomy to the date of death or the date of the last follow-up (either a clinical visit or a discussion with the patient's referring physician); deaths due to any cause were considered to represent treatment failures. Time to recurrence was calculated from cystectomy to the date of the first documented clinical recurrence or the last follow-up; data on patients who died free of disease before any recurrence were censored at the time of death. One patient was known to have died of disease, but the date of recurrence was unknown and therefore a date midway between the last clinic visit and the date of death was used. For the purpose of the statistical analysis, nuclear accumulations of p53 were classified as either positive (accumulation in 10 percent or more of tumor cells) or negative.

Contingency tables, Pearson's chi-square test, and logistic regression[17] were used to evaluate the association of p53 accumulation with lymph-node status, pathological stage of the primary tumor, and histologic grade. Kaplan–Meier plots[18] and the log-rank test[19] were used to evaluate the association of these three standard clinical prognostic variables, as well as the expression of p53, with the time to recurrence and with survival. Greenwood's formula[19] was used to estimate the standard errors of the Kaplan–Meier estimates of the probability of survival or recurrence. To determine whether an accumulation of p53 provided prognostic information beyond that provided by the three standard clinical variables, a stratified log-rank test was used. All P values reported are two-sided.

The statistical analyses were performed for the entire cohort of 243 patients and were performed separately for the 190 patients not previously tested for accumulation of p53.

Results

Of the 190 bladder tumors not previously examined for accumulation of p53, 112 had no nuclear reactivity and 7 had a few focally positive tumor nuclei but less than 10 percent. These 119 tumors were considered p53-negative. In 54 of the remaining 71 tumors, p53 protein was detected in 10 to 49 percent of tumor-cell nuclei, and in 17 tumors p53 was seen in 50 to 100 percent of tumor-cell nuclei. These 71 bladder cancers were considered p53-positive. In all 243 patients, 142 tumors were classified as p53-negative and 101 as p53-positive.

Association of p53 Nuclear Reactivity with Tumor Grade, Pathological Stage, and Lymph-Node Status

Analysis of the 190 tumors not previously tested for p53 revealed that nuclear p53 was identified more frequently in grade 4 tumors than in grade 2 or 3 tumors, but this association was not statistically significant ($P = 0.20$) (Table 1). By contrast, the presence of nuclear p53 was significantly associated with pathological stage ($P = 0.003$); 32 percent of the patients with negative lymph nodes, as compared with 55 percent of the patients with positive lymph nodes, had nuclear accumulation of p53 ($P = 0.008$). In patients with no evidence of lymph-node metastases, the presence of nuclear p53 was associated with greater depth of invasion; 22 percent of the patients with superficial disease (stage Pa, Pis, or P1), as compared with 38 percent of the patients with disease invasive of muscle (stages P2 through P4), had nuclear p53 in the tumor cells ($P = 0.044$). Similar results were obtained for the entire cohort of 243 patients.

Association of p53 Immunoreactivity with Recurrence and Overall Survival

In the group of 190 patients not previously tested for p53 alterations, nuclear accumulation of p53 was significantly associated with an increased probability of tumor recurrence ($P<0.001$) and a decreased probability of survival ($P<0.001$). Identical results were obtained for the entire cohort of 243 patients; Figures 2 and 3 show curves for recurrence and survival for all patients.

Among the patients with no evidence of lymph-node involvement, nuclear accumulation of p53 was significantly associated with recurrence in those with disease confined to the bladder (stage P1, P2, or P3a). However, this association was not statistically significant among the patients with extravesical extension of

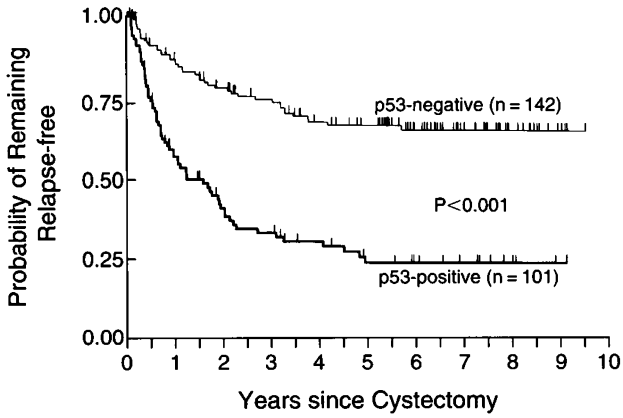

Figure 2. Probability of Remaining Relapse-free in 243 Patients with Bladder Cancer and either p53-Positive or p53-Negative Tumors.

Identical results were obtained for the subgroup of 190 patients not previously tested for p53 alterations. Each tick mark represents a patient who had not had a recurrence of disease at the time of the last follow-up.

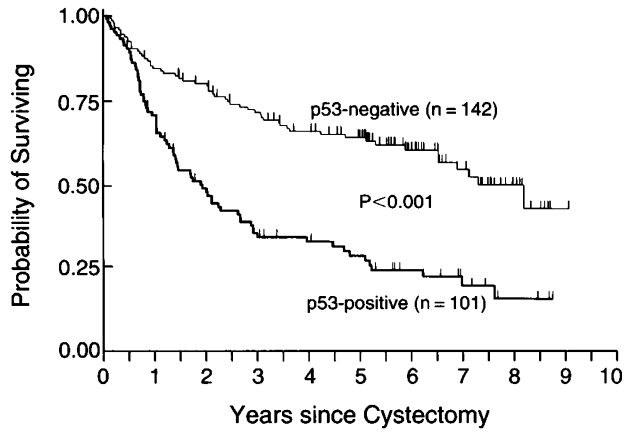

Figure 3. Probability of Survival in Patients with Bladder Cancer and either p53-Positive or p53-Negative Tumors.

Each tick mark represents a patient who was alive at the time of the last follow-up.

Table 2. Estimated Rates of Recurrence and Survival at Five Years in 190 Patients with Bladder Cancer, According to p53 Status of Tumors and Pathological Stage.*

Group and Stage	No. of Patients	Rates of Recurrence			Rates of Survival		
		p53-negative	p53-positive	P value	p53-negative	p53-positive	P value
		percent			percent		
Lymph nodes negative							
Confined to bladder							
Pa or Pis	15	18±12	33±27	0.48	75±13	67±27	0.73
P1	39	7±5	62±17	0.002	93±5	78±14	0.004
P2	29	12±8	56±17	0.007	79±10	30±15	0.023
P3a	20	11±11	80±17	0.011	64±15	22±14	0.20
Not confined to bladder							
P3b	36	59±11	73±12	0.23	52±11	27±11	0.18
P4	9	43±19	100	0.20	57±19	0	0.12
Lymph nodes positive	42	69±12	91±6	0.054	32±11	13±7	0.19

*Rates of recurrence and survival are based on Kaplan–Meier estimates[18]; plus–minus values are estimates of the standard error, calculated with Greenwood's formula.[19] Tumors considered to be p53-negative had less than 10 percent of tumor-cell nuclei demonstrating p53 immunoreactivity, and those considered to be p53-positive had 10 percent or more of tumor-cell nuclei demonstrating p53 immunoreactivity.

tumor (stage P3b or P4) (Table 2). The five-year recurrence rates for patients with stage P1, P2, and P3a tumors that were p53-negative were 7, 12, and 11 percent, respectively. In contrast, the five-year recurrence rates were 62, 56, and 80 percent for stage P1, P2, and P3a tumors that were p53-positive (Table 2). In all subgroups of tumors confined to the bladder, the recurrence rates at five years for p53-negative and p53-positive tumors differed significantly. Significant differences in estimated five-year survival rates were also found among patients with tumors confined to the bladder (except stage P3a tumors) (Table 2). Identical results were obtained for the entire cohort of 243 patients. Figures 4 and 5 show the Kaplan–Meier curves for recurrence and survival in all 243 patients, stratified according to the depth of tumor invasion.

In a multivariable analysis stratified according to grade, pathological stage, and presence or absence of lymph-node metastases nuclear p53 status was an independent predictor of the recurrence of bladder cancer and of overall survival ($P<0.001$). When patients with lymph-node–negative cancers confined to the bladder were stratified according to p53 status, pathological stage (i.e., depth of invasion) was not an independent predictor of either recurrence ($P = 0.62$) or survival ($P = 0.23$).

Forty-one of the 243 patients received postoperative adjuvant systemic chemotherapy. When these patients were removed from the analysis, the presence of nuclear p53 remained highly associated with recurrence and death. This analysis demonstrates the predictive value of nuclear p53 reactivity in a cohort of patients uniformly treated with radical cystectomy and pelvic-lymph-node dissection but no adjuvant therapy.

Discussion

We found that the immunohistochemical detection of p53 protein in the nuclei of tumor cells can provide prognostic information in patients with bladder cancer. In patients with transitional-cell carcinoma of the bladder treated by cystectomy, the nuclear accumulation of p53 correlated with a significantly increased risk of recurrence and decreased overall survival. The link between nuclear p53 and prognosis was independent of tumor grade, pathological stage, and lymph-node status. The strongest association between p53 immunoreactivity in tumor-cell nuclei and tumor progression was observed when the disease was confined to the bladder. The presence of nuclear p53 was strongly associated with an increased risk of recurrence among patients with P1, P2, or P3a disease and with decreased overall survival among those with P1 or P2 disease. It was the only independent predic-

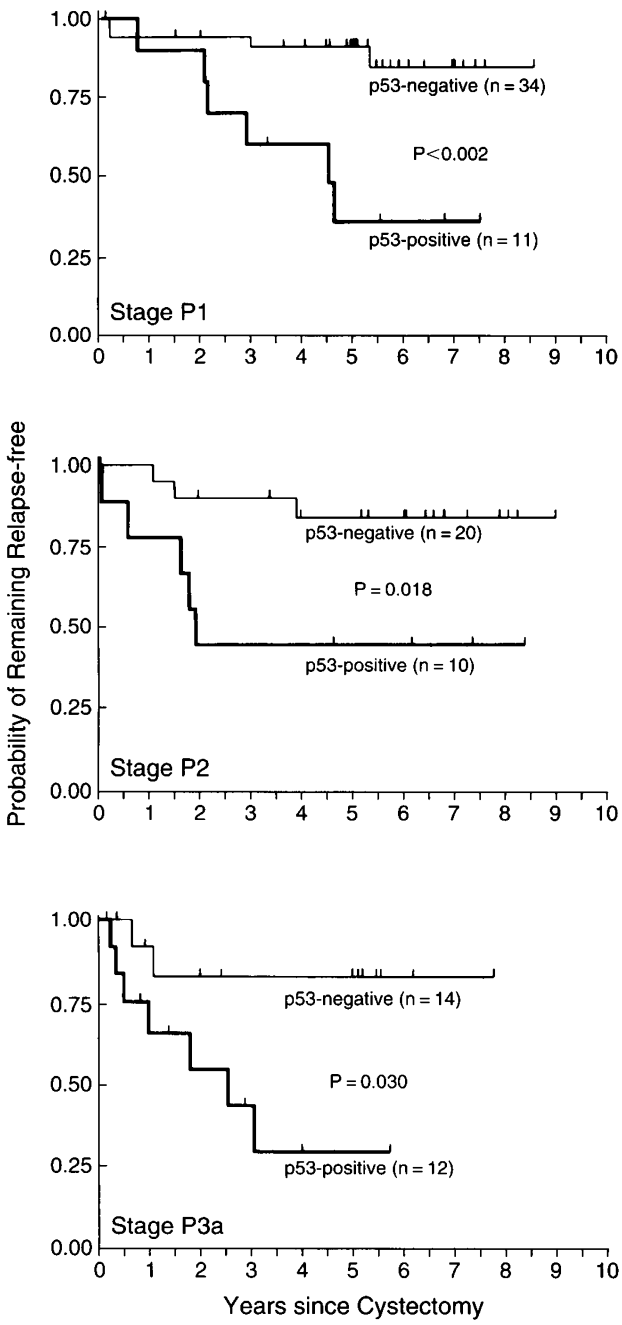

Figure 4. Probability of Remaining Relapse-free According to Pathological Stage in Patients with Organ-Confined Bladder Cancer but No Regional Lymph-Node Metastases.

All patients with P1, P2, or P3a disease from the entire cohort of 243 patients were included in this analysis. Identical results were obtained for the patients with P1, P2, or P3a disease in the subgroup of 190 patients not previously tested for p53 alterations.

tor of disease progression in a multivariable comparison of p53 status, pathological stage, and histologic grade.

The product of a mutated p53 gene can be a metabolically stable protein with a long half-life.[20] In contrast, wild-type p53 has a short half-life, only 6 to 30 minutes, and thus it does not generally accumulate in high enough levels to be detected by standard immunohistochemical methods. A long-lived mutated p53 protein, in contrast, is usually detectable by such techniques. We have previously demonstrated a significant association of p53 mutations with the immunohistochemical detection of p53 protein in tumor-cell nuclei.[5] Immunohistochemical techniques identified most cases in which the cells had mutated p53 genes, but 15 to 20 percent of tumors with demonstrable mutations of the p53 gene were negative by immunohistochemical analysis.[5] Moreover, a substantial proportion of tumors with nuclear accumulation of p53 lacked evidence of p53 gene mutation. Alterations that render the p53 protein detectable by immunohistochemical analysis may not require a mutation in the p53 gene. For example, cellular oncogene products that bind to and inactivate wild-type p53 protein, such as MDM2,[21] may prolong the half-life of p53, allowing it to accumulate in the nucleus. Although this phenomenon has not been shown directly, recent studies indicate that cells that overexpress MDM2 can also overexpress p53 protein in the absence of a p53 gene mutation.[22] Moreover, the level of wild-type p53 protein can increase in response to DNA damage.[23] Thus, increases in the level of the p53 protein and mutation of the p53 gene occur together in most cases but can be separate events. Although we have shown that the presence of p53 alterations as detected by immunohistochemical methods is clinically relevant, it may also be important to assess mutations of the p53 gene directly as a prognostic indicator in bladder cancer.

The most important finding of this study is that in patients with transitional-cell carcinoma confined to the bladder (stages P1, P2, and P3a), and no evidence of lymph-node metastases, p53 nuclear reactivity identifies tumors with a tendency to progress. In these cases, the detection of nuclear p53 is more strongly associated with tumor recurrence and decreased survival than is the depth of invasion or histologic grade. Patients with p53-negative tumors had relatively low rates of recurrence, whereas metastases developed in the majority of patients with p53-positive tumors, regardless of depth of invasion. Although depth of invasion is clearly associated with the progression of disease in patients with bladder cancer, this may reflect the proportion of tumors in each stage that have p53 alterations.

This study may influence the selection of patients for adjuvant treatment of bladder cancer, which currently depends on the depth of tumor invasion and the detection of regional lymph-node metastases. The results of two recent studies are encouraging,[11,12] but the role of adjuvant chemotherapy after cystectomy, particularly in patients with disease confined to the bladder, remains unresolved. Patients with p53-negative tumors confined to the bladder have a low rate of disease progression (even when there is deep muscle invasion) and may not require adjuvant treatment after radical cystectomy. In contrast, patients with p53-positive cancer confined to the bladder (including those with only superficially invasive tumors) have a poor prognosis. They may benefit from adjuvant treat-

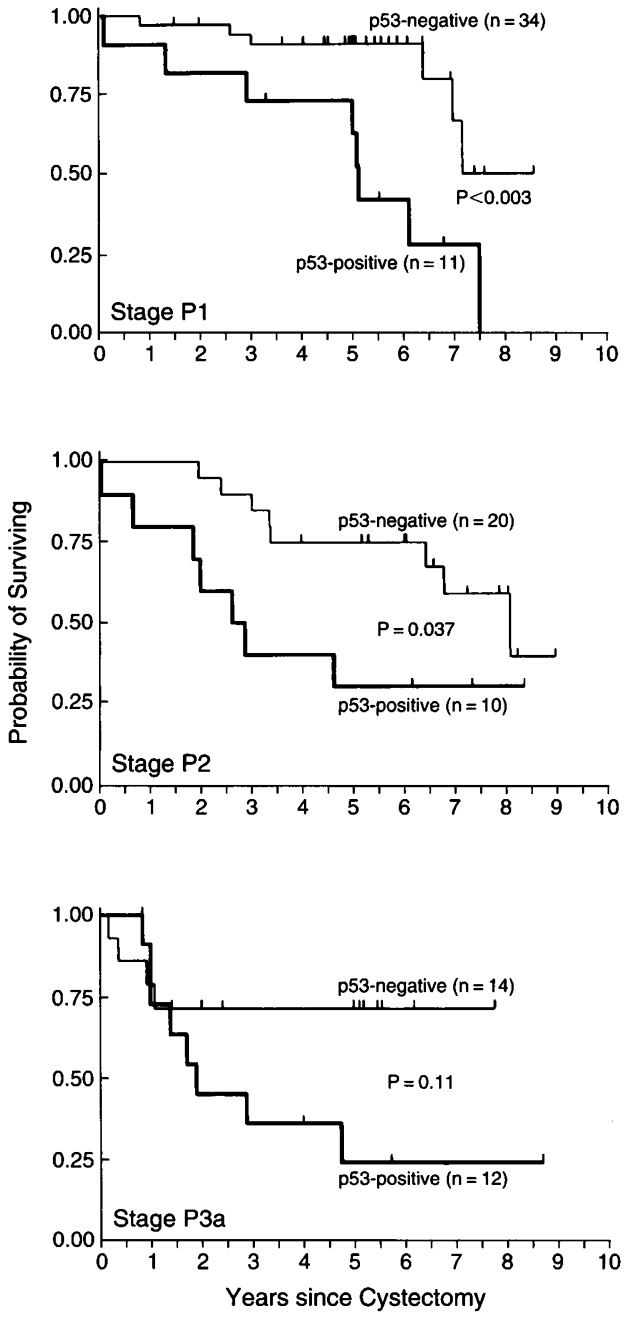

Figure 5. Probability of Survival According to Pathological Stage in Patients with Organ-Confined Bladder Cancer but No Regional Lymph-Node Metastases.

ment. This study and the study by Sarkis et al.[13] are also relevant to the controversial issue of surgical treatment of superficially invasive bladder cancer. Patients with superficially invasive p53-positive tumors have a high rate of disease progression and may therefore benefit from early radical cystectomy. Moreover, our study indicates that patients with p53-positive tumors are at high risk of progression despite radical cystectomy and may therefore benefit from adjuvant treatment. Thus, the immunohistochemical detection of p53 may identify patients with cancer confined to the bladder who could benefit from radical surgery and adjuvant therapy. Furthermore, the absence of detectable p53 may be an indication for conservative therapy, even in the presence of locally advanced disease.

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