

Inactivation of the p53 Pathway in Prostate Cancer: Impact on Tumor Progression¹

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ABSTRACT

To determine the potential role of p53 inactivation in prostate cancer, we studied a well characterized cohort of 86 patients treated with radical prostatectomy. We analyzed patterns of p53, mdm2, and p21/WAF1 expression by immunohistochemistry. Results were then correlated with clinicopathological parameters of poor outcome, including time to PSA relapse. In addition, data were also correlated with proliferative index, as assessed by Ki67 antigen detection. p53-positive phenotype, defined as identification of nuclear immunoreactivity in >20% of tumor cells, was observed in 6 of 86 cases (7%). An association was observed between p53-positive phenotype and decreased time to PSA relapse ($P < 0.01$). mdm2-positive phenotype, defined as $\geq 20\%$ of tumor cells displaying nuclear immunoreactivity, was observed in 28 of 86 cases (32.5%). mdm-2-positive phenotype was found to be associated with advanced stage ($P = 0.009$). p21-positive phenotype, defined as >5% of tumor cells with nuclear immunoreactivity, was observed in 28 of 86 cases (32.5%). An association was observed between p21-positive phenotype and high Ki67 proliferative index ($P = 0.002$). Patients with p21-positive phenotype had a significant association with decreased time to PSA relapse ($P = 0.0165$). In addition, a significant association was found between p21-positive phenotype and coexpression of mdm2 ($P < 0.01$). Forty-three of 86 cases (50%) were found to have one or more alterations, and patients with any alteration were found to have a higher rate of PSA relapse ($P < 0.01$). It is our hypothesis that a pathway of prostate cancer progression involves p53 inactivation caused by mdm2 overexpression and that p21 transactivation in this setting is due to an alternative signaling system, rather than through a p53-dependent mechanism.

Received 2/8/99; revised 5/10/99; accepted 5/17/99.

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¹ Supported in part by NIH Grant CA-DK-47650 (to C. C.-C.) and the PepsiCo. Foundation (to M. D. and H. I. S.).

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INTRODUCTION

p53 responds to different forms of cellular stress by targeting and activating genes involved in growth arrest and cell death. A target of p53-induced transcription is the *p21/WAF1* gene, which encodes a cyclin-dependent kinase inhibitor (1). In addition, levels of p53 are tightly regulated by mdm2, which binds to p53, repressing its activity and triggering its degradation. The *MDM2* gene is itself under the transcriptional control of p53, creating an autoregulatory feedback loop (2).

Alterations in the *TP53* gene seem to be uncommon in prostate cancer, and their clinical significance has not been fully investigated. A recognized limitation of most studies is that they are confined to the analysis of p53 alterations, without analyzing other critical components that regulate its functions. The *MDM2* gene is amplified in a variety of tumors, and mdm2 overexpression without amplification seems to be a common mechanism of p53 inactivation in certain cancers (3, 4). Lack of data regarding the functional status of the p53 products encountered in the tumors analyzed represents another drawback. It has been reported that *p21/WAF1* gene expression may serve as an indicator of p53 activity because *p21/WAF1* is under the transcriptional control of p53. However, serum or individual growth factors, such as epidermal growth factor and fibroblast growth factor, were shown to induce p21 expression in p53-deficient cells (5, 6). Thus, there are at least two separate pathways accounting for the induction of p21, one linked to DNA-damage recognition, and the other produced by signaling mechanisms caused by certain cellular mitogens.

In the present study, we have analyzed the patterns of p53 expression and those of critical components of its pathway, namely mdm2 and p21, in 86 patients with prostate cancer. The association between these markers and clinicopathological parameters of poor outcome, including time to PSA³ relapse and proliferative index, were also examined.

MATERIALS AND METHODS

Patients. A total of 86 patients who underwent radical prostatectomy at Memorial Sloan-Kettering Cancer Center in the period between 1990 through 1991 were studied. Patient selection was based on the availability of both adequate clinical follow-up and representative archival pathological materials for immunohistochemical analysis. The median age at the time of surgery was 65 years (range, 46–74). Their median follow-up was 64.5 months (range, 10–94). Formalin-fixed, paraffin-embedded prostate tissues were obtained from our archival tumor bank. Representative H&E stained sections were examined to evaluate the histopathological characteristics of each case.

Clinicopathological parameters examined include pretreat-

³ The abbreviation used is: PSA, prostate-specific antigen.

ment PSA, pathological stage, and Gleason score, both determined based on the radical prostatectomy specimen. Time to PSA relapse was calculated from the day of surgery to the first detectable PSA. PSA relapse was defined as three consecutive rises in PSA at least 1-week apart. Only patients who had an undetectable PSA level after surgery were included in this analysis.

Tumors were staged pT2 ($n = 51$) and pT3 ($n = 35$). Twenty-nine patients were Gleason score <7 , whereas 18 patients were Gleason ≥ 7 . In six cases, due to scarcity of tumor representation in the specimen, grade was considered to be not interpretable. Thirty-three patients (38.3%) received neoadjuvant hormone treatment preoperatively and were defined as hormone treated. These patients had nonevaluable Gleason scores. Patients who did not receive neoadjuvant hormone treatment were defined as hormone-naive.

Monoclonal Antibodies and Immunohistochemistry.

The following well characterized mouse monoclonal antibodies and corresponding final working dilutions were used for the present study: anti-p53 monoclonal antibody PAB1801 (Ab-2 clone; CalBiochem/Oncogene Science, Boston, MA; 1:500 dilution); anti-mdm2 monoclonal antibody 2A10 [a gift from Dr. Arnold Levine (Rockefeller University, New York, NY); 1:500 dilution]; and an anti-p21 monoclonal antibody (Ab-1 clone; CalBiochem/Oncogene Science, 1:20 dilution). An anti-Ki67 mouse monoclonal antibody (clone MIB1; Immunotech SA, France; 1:50 dilution) was used to assess proliferative index. MlgS-Kp1, a mouse monoclonal antibody of the same subclass as the primary antibodies listed above was used as negative control.

An avidin-biotin immunoperoxidase method was used. Briefly, sections were subsequently immersed in boiling 0.01 M solution of citric acid, adjusted to pH 6.0 for 15 min to enhance antigen retrieval, and incubated with primary antibodies overnight at 4°C. Biotinylated horse antimouse IgG antibodies were applied for 1 h (Vector Laboratories, Burlingame, CA; 1:500 dilution), followed by avidin-biotin peroxidase complexes for 30 min (Vector Laboratories; 1:25 dilution). Diaminobenzidine was used as the final chromogen, and hematoxylin was used as the nuclear counterstain. Nuclear immunoreactivity were classified on a continuous scale with values that ranged from undetectable levels or 0% to homogeneous staining or 100%.

Statistical Analysis. The three markers were analyzed both as percentage of tumor cells and as discrete variables based on *a priori* cut-points. The cut-point for p53 of $>20\%$ was based on our previous analysis of p53 alterations in bladder cancer that revealed a strong association between p53 point mutation and p53 nuclear accumulation in $>20\%$ of tumor cells (7, 8). For mdm2, the cut-point was based on what has been published, correlating mdm2 overexpression in $\geq 20\%$ of tumor cells with worse clinicopathological parameters (9, 10). The same principle applied to the Ki67 $>20\%$ cut-point determination (11, 12). For p21, the cut-point of $>5\%$ was based on our finding that normal prostate glands lack p21 expression and the observation of p21 nuclear staining and presence of mitotic figures indicating high proliferative activity of the tumors.

The association of percentage of tumor cells expressing the markers with time to PSA relapse, while adjusting for other variables with known prognostic significance, was assessed

using the Cox proportional hazards model (13). In addition, Kaplan-Meier estimation (14) was performed, and the log rank test (15) was used to assess the univariate relationship between the individual markers using cut-points and time to PSA relapse.

The associations between Gleason group and the three biomarkers were assessed using Fisher's exact test (16). Also, associations between the three markers and variables such as Ki67 proliferative index, stage, and hormone status were also assessed using the above test.

RESULTS

Table 1 summarizes the data in relation to clinicopathological parameters, including pretreatment, PSA, tumor stage, Gleason tumor grade, hormone status, proliferative index, and immunophenotype profile. Fig. 2 illustrates the univariate relationships of the three markers with time to PSA relapse with Kaplan-Meier curves estimated.

p53 nuclear overexpression of $>20\%$ was observed in 6 of 86 cases (Fig. 1). The distribution of p53 % expression was primarily patients expressing $<5\%$ p53 ($n = 76$). The other 10 patients had varying levels of p53 percentage expression, indicating a very low frequency of p53 alteration in this group of patients. There is no correlation between p53-positive phenotype and pretreatment PSA, tumor stage, tumor grade, hormone status, or high proliferative index. Also, there is no association between p53 overexpression and p21 or mdm2 overexpression. A significant association was observed between p53 status, determined by the cut-point and time to PSA relapse. This association is illustrated in Fig. 2. Using the log rank test to examine the overall differences between p53-negative phenotype and p53-positive phenotype revealed a statistical significant difference $P < 0.01$. This indicates an obvious PSA relapse time advantage for patients who do not overexpress p53. However, the magnitude of this difference may not be reliably estimated due to the small number of patients and events in the p53-positive phenotype group.

mdm2 nuclear overexpression of $\geq 20\%$ tumor cells was observed in 28 of 86 cases (32.5%) (Fig. 1). mdm2-positive phenotype was associated with advanced stage ($P = 0.009$). In addition, mdm2 overexpression was observed not to be significant with respect to a decreased time to PSA relapse (Fig. 2). A trend was observed between mdm2 overexpression and higher pretreatment PSA ($P = 0.06$).

p21 nuclear overexpression of $>5\%$ of tumor cells was observed in 28 of 86 patients (32.5%) (Fig. 1). Patients with p21-positive phenotype were observed to have a significant association with high Ki67 proliferative index ($P = 0.002$). High Ki67 proliferative index was identified in 11 of 86 patients (12.7%) (Fig. 1). Patients with p21-positive phenotype had a significant association with decreased time to PSA relapse, as illustrated in Fig. 2. Also, p21 overexpression was associated with mdm2 overexpression ($P < 0.01$). However, no association was observed between identification of p21- and/or mdm2-positive phenotype and p53 overexpression.

Forty-three of the total 86 patients had one or more altered markers. Patients with any alteration (p53 or mdm2 or p21) were observed to have a higher rate of PSA relapse ($P < 0.01$).

The multivariate relationship between the markers and time

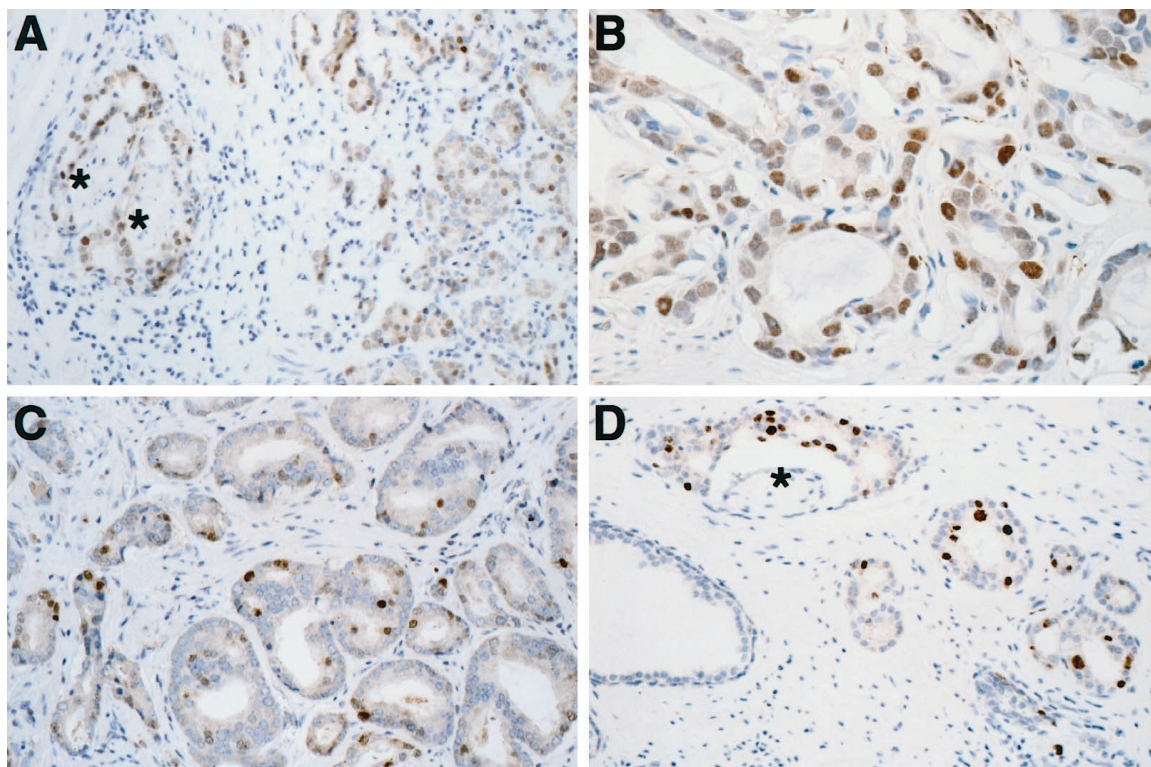


Fig. 1 Photomicrographs of selected primary prostate carcinoma cases analyzed by immunohistochemistry using mouse monoclonal antibodies PAB1801 (anti-p53; **A**), 2A10 (anti-mdm2; **B**), Ab-1 (anti-p21; **C**), and MIB1 (anti-Ki67; **D**). **A**, p53 nuclear overexpression in tumor cells. Note the positive nuclear staining of tumor cells at a perineural invasion site (*). **B**, mdm2 nuclear overexpression in tumor cells. **C**, p21 nuclear overexpression in tumor cells. **D**, high Ki67 proliferative index. Note the intense Ki67 nuclear staining of tumor cells at a perineural invasion site (*).

to PSA relapse was assessed using Cox proportional hazards model. It was of interest to examine the effect of the markers while adjusting for variables with no prognostic significance. Both, p53- and p21-positive phenotypes were significant while adjusting for pretreatment PSA and Gleason group ($P < 0.01$ for both markers). Examination of the overexpression of at least one marker (p53, mdm2, or p21) with respect to time to PSA relapse showed that this variable was also significant ($P < 0.01$) while adjusted for pretreatment PSA and Gleason group. Tumor stage (<3 versus ≥ 3) was not significant in either the univariate or multivariate analyses and was, thus, excluded from the model. The model that seemed to account for the most information included p53 and p21, along with pretreatment PSA.

DISCUSSION

Studies dealing with the frequency of TP53 mutations and p53 overexpression in prostate cancer have yielded conflicting results, alterations ranging from 2–65% of cases studied (17–21). This discrepancy might be explained by the relatively small number of cases and different disease stages analyzed in some studies, the distinct methodologies used, and the cutoff points used for evaluation of immunohistochemical results. However, a general finding was the association between p53 alterations and clinicopathological parameters of poor clinical outcome, such as high grade and late stage (18, 19, 22). In this study, we observed a relatively low frequency of p53 nuclear overexpression in

patients with localized prostate cancer, as previously reported (23, 24). To determine the potential clinical relevance of identifying a p53-positive phenotype, we correlated phenotypic characteristics of the tumors with the time to PSA relapse. This is considered the most sensitive indicator of success or failure after radical prostatectomy in patients treated for localized disease. Analysis of data revealed that p53 overexpression was significantly associated with PSA relapse ($P < 0.01$) and independent of pretreatment PSA and Gleason group. However, the magnitude of this difference may not be reliably estimated due to the small number of patients and events in the positive phenotype. We also observed that all patients who received neoadjuvant hormone treatment before surgery and had tumors that overexpressed p53 relapsed. This finding could be due to the advanced stage at which patients presented and were selected for treatment using this modality. Mechanistically, an altered p53 status in this setting could have conferred resistance to castration-induced apoptosis, ultimately leading to disease relapse. The association between p53 overexpression and hormone refractory prostate cancer has been reported in locally advanced and metastatic disease (25). However, to our knowledge, this is the first study to suggest that this association might be an early event in the evolution of hormone refractory disease in clinically localized prostate cancer.

In the present study, we also analyzed alterations affecting other regulators of the p53 pathway in primary prostate cancer,

Table 1 Summary of data in relation to immunophenotype profile

	p53		p21		mdm-2	
	n	%	n	%	n	%
PSA						
<4	0/18	0	5/18	27	3/18	16
4–10	3/28	10	7/28	25	7/28	25
>10	3/40	7.5	16/40	40	18/40	45
P		0.374		0.382		0.060
Stage						
T < 3	3/51	5.8	14/51	27	11/51	21
T = 3	3/35	8.5	14/35	40	17/35	48
P		0.631		0.222		0.009
Gleason score						
<7	0/29	0	7/29	24	7/29	24
=7	2/18	11	10/18	22	9/18	50
NE ^a	4/33	2	10/33	30	11/33	33
P		0.157		0.074		0.190
Hormone status						
Naive	2/53	3	18/53	33	17/53	32
Treated	4/33	12	10/33	30	11/33	33
P		0.139		0.725		0.904
Proliferation index Ki67						
low	5/75	6	20/75	26	22/75	29
High	1/11	9	8/11	72	6/11	54
P		0.768		0.002		0.096

^a NE, not evaluable.

including mdm2 and p21. The *MDM2* gene maps to 12q13 and is found overexpressed in certain tumors due to its amplification as a component of an amplicon that includes other relevant genes, such as CDK4. The *MDM2* gene is under transcriptional regulation by p53 and encodes a 90-kDa zinc finger protein (mdm2) that contains a p53 binding site (26). It has been shown that mdm2 binds to p53 and acts as a negative regulator by inhibiting p53 transcriptional activity and targeting its degradation, thus creating an autoregulatory feedback loop (27). In this study, nuclear mdm2 overexpression was found in 32.5% of cases. We observed that mdm2-positive phenotype was significantly associated with advanced stage. It has been previously reported that *MDM2* is not amplified on primary prostate cancer, based on a study of 29 tumors analyzed by Southern blot hybridization (28). The discrepancy between the rate of *MDM2* gene amplification and protein overexpression has been described in Burkitt's lymphoma and breast cancer (29, 30). Furthermore, it was observed in soft tissue sarcomas that mdm2 overexpression, rather than its amplification, was associated with worse clinical outcome (10). On the basis of data from this study, we can postulate that mdm2 overexpression is a frequent mechanism of p53 inactivation in prostate cancer and, in this context, the *MDM2* gene can be classified as an oncogene in this setting.

The *p21/WAF1* gene encodes a nuclear protein member of the cyclin-dependent kinase inhibitory KIP family involved in senescence and cell quiescence (31). The *p21/WAF1* gene is also transcriptionally regulated by p53. However, p21 induction could also be accomplished by a p53-independent pathway. Serum or individual growth factors, such as epidermal growth factor and fibroblast growth factor, were shown to induce p21 in p53-deficient cells (32). On the basis of these data, it has been postulated that p21 induction could be activated through two

separate pathways. The rate of p21/WAF1 mutations in human cancer is very low (33). However, there is an association between altered patterns of p21 expression and clinical outcome in certain tumors, such as bladder, colon, and hepatocellular carcinomas (34–36). Lack of p21 expression in these studies was correlated with poor clinical outcome, an expected finding if one postulates that p21 deficiency reflects p53 inactivation. As a corollary to this hypothesis, the p21-negative phenotype observed in the above referred studies was usually associated with p53 alterations. However, in our study, we found that p21-positive phenotype was significantly associated with high proliferative index and mdm2 overexpression, but not with p53 status. Moreover, patients with p21-positive phenotype had a significant association with decreased in time to PSA relapse. p21 overexpression has been reported to be associated with worse prognosis in other tumor types, including breast and esophageal carcinoma, and squamous cell carcinomas of the head and neck (37–39). Moreover, p21 overexpression was found to be associated with resistance to chemotherapy in acute myeloid leukemia and glioblastoma (40, 41).

These data could be interpreted as follows (see Fig. 3). A positive p21 phenotype could signify activation of p53 in response to DNA damage or cellular stress. This effect would result in G₁ arrest of the prostate tumor cells expressing p21. We observed, on the contrary, an association between p21-positive phenotype and increased proliferative activity. Thus, it is more plausible to postulate that the p21 overexpression observed is caused by a p53-independent transactivation mechanism. In the setting of prostate cancer, the alternative mechanism could be due to mitogenic stimuli via growth factor signaling. There is abundant evidence regarding the up-regulation of growth factor receptor/ligand activity in prostate tumors (42–46). An additional aberration causing p53 inactivation would be required in

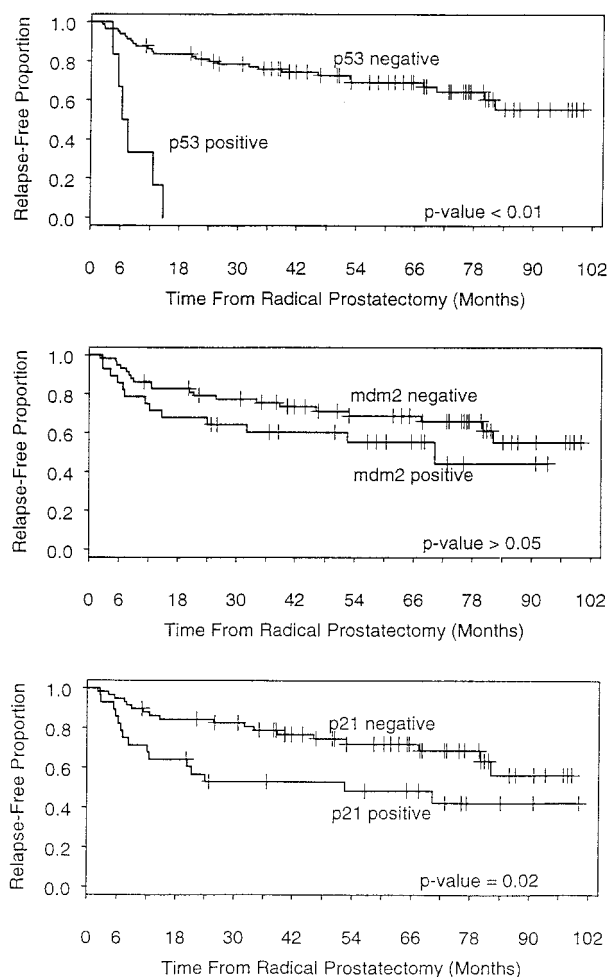


Fig. 2 Progression-free and survival curves for patients with primary prostate cancer. The Kaplan-Meier method was used to estimate overall disease-free survival. The log-rank analysis was used to compare the different curves. *A*, progression was significantly reduced in patients with tumors displaying a p53-positive phenotype ($P < 0.01$). *B*, progression was not related to mdm2 status. *C*, progression was significantly reduced in patients with tumors displaying a p21-positive phenotype ($P = 0.0165$).

this model to explain the lack of cell death and association with proliferative activity. It is our hypothesis that the increased mdm2 expression discussed above provides this requirement, further supporting the oncogenic role of mdm2 in prostate cancer.

Finally, the association between p21 and high proliferative index might also reflect deregulated cyclinD1/CDK4 activity. In fact, we observed a strong association between p21-positive phenotype and cyclin D1 overexpression in this cohort of patients.⁴ Taken together, these data support the concept that p21 overexpression denotes an inefficient pRB control on S-phase entry.

⁴ M. Drobnjak *et al.*, personal communication.

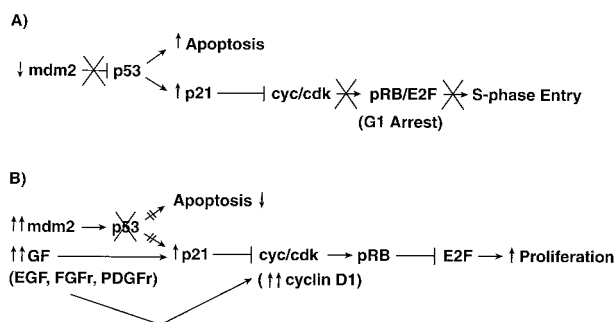


Fig. 3 Diagrammatic representation of the p53 pathway (*A*) and alterations that may develop during tumor progression in prostate cancer (*B*). *A*, p53 regulates the expression of several genes involved in cell cycle arrest (*i.e.*, p21) and apoptosis (*i.e.*, bax). p21 binds to heterodimeric protein kinases formed by cyclins and cyclin-dependent kinases (*Cdks*), blocking phosphorylation of pRB/E2F1 complexes and abrogating S-phase entry. p53 also produces an autoregulatory feedback loop by transactivating mdm2. *B*, overexpression of mdm2 has been observed to occur in several tumor types, and it is considered an oncogenic event. On binding to mdm2, p53 products are transcriptionally inactivated and triggered for degradation. This will release the G₁ arrest imposed, in part, by p21 and abolish the apoptotic signals of the pathway. Thus, inactivation of p53 will favor proliferative activity, immortality, and development/accumulation of further DNA damage or mutations. The increased p21 expression observed in our study could be produced via growth factor signaling, which would also impact on cyclin D1 expression. The increment of p21 does not seem to be able to control the proliferative activity of tumor cells, as attested by the association of p21-positive phenotype and high Ki67 proliferative index. Taken together, mdm2 overexpression will inactivate the p53 pathway, whereas increased mitogenic activity will offset the RB pathway. The mechanistic basis for this dual requirement stems, in part, from the deactivation of a p53-dependent cell suicide program that would normally be brought about as a response to unchecked cellular proliferation resulting from RB deficiency.

Growth control in mammalian cells is accomplished largely by the action of the RB protein, regulating exit from the G₁ phase and the p53 protein, triggering growth arrest or apoptotic processes. In this group of patients, there is enough evidence to suggest that both mechanisms are defective in prostate cancer. The high proliferative index reflects the inefficient pRB control. We postulate that this phenomenon is produced by deregulated cyclin D1/CDK4 activity, which is associated with a p21 positive phenotype. The deactivation of a p53-dependent apoptosis could be explained by the degradation of p53 induced by mdm2 overexpression.

In summary, alterations affecting the p53 pathway are frequent events in prostate cancer. It is our hypothesis that a pathway of prostate cancer progression involves p53 inactivation caused by mdm2 overexpression, and that p21 transactivation in this setting is due to an alternative signaling system rather than through a p53-dependent mechanism.

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