

The Association of p21^(WAF-1/CIP1) with Progression to Androgen-independent Prostate Cancer¹

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ABSTRACT

The molecular events leading to progression toward androgen-independent prostate cancer (AIPC) are not fully understood. The p21^(WAF-1/CIP1) (p21) gene has been identified as a key factor for the regulation of cell growth. The expression of p21 was examined by immunohistochemical studies in 105 prostate cancer samples: (a) 7 of 30 (23%) androgen-dependent tumors; and (b) 36 of 75 (48%) androgen-independent tumors stained positive for p21 ($P < 0.02$). No association was found between p21 expression and p53, bcl-2, and the androgen receptor protein expression in bone metastases of patients with AIPC, whereas there was a significant association with a high Ki-67 index ($P < 0.05$). In 4 of 43 (9%) cases, tumors displayed a p53-negative, bcl-2-negative, and p21-positive phenotype. A xenograft mouse model of prostate cancer using the androgen-responsive MDA PCa 2b prostate cancer cell line was used to study p21 expression after androgen deprivation and at relapse. Androgen deprivation reduced p21 expression to undetectable levels after 14 days. Tumor relapse, defining AIPC, was associated with increased expression of p21 to levels comparable with those found before castration. In this model, p21 expression at relapse was also correlated with a high Ki-67 index. In conclusion, p21 expression is associated with the progression to AIPC. A possible explanation involves a

paracrine effect of p21 mediated by the release of mitogenic and antiapoptotic factors. Another explanation involves the regulation of p21 expression by the androgen receptor, which also suggests that p21 may have antiapoptotic function in prostate cancer.

INTRODUCTION

Prostate cancer is the most common cancer and the second leading cause of death by cancer among men in the United States (1). Although localized prostate cancer is curable by a variety of treatments, including radical prostatectomy, radiotherapy, and brachytherapy, the treatment of disseminated disease is only palliative. Although the disease is characterized initially by a high sensitivity to androgen deprivation, most deaths result eventually from the progression from an androgen-dependent to an androgen-independent status. Indeed, there is no available standard treatment for AIPC,³ and the molecular basis of androgen independence is still incompletely understood (2).

It is likely that prostate cancer cells can achieve the transition to androgen-independent growth by different multistep routes, including inhibition of apoptosis and bypassing or adapting the androgen receptor pathway (3–5). Prostate cancer cells can survive in an environment with low-androgen levels by altering the androgen receptor pathway in a number of ways and usually keeping it functional. These include mutations that alter ligand specificity leading to activation by progesterone, estrogen, glucocorticoids, or HER-2/*neu* (5, 6); ligand-independent activation of androgen receptors by growth factors and cytokines, at least *in vitro* (3, 5); androgen receptor amplification, which is found in ~30% of cases (5); and possibly steroid receptor coactivator amplification, corepressor down-regulation, or both (3). Alternatively, although it is believed that the androgen receptor pathway remains active in AIPC, a bypass of ligand-dependent activation of the androgen receptor in prostate cancer cells could be produced by growth-stimulating factors acting in an autocrine/paracrine fashion, including epidermal growth factor, insulin-like growth factor-I and-II, transforming growth factor α , keratinocyte growth factor, and fibroblast growth factors (reviewed in Ref. 3).

Besides the mechanisms involving the androgen receptor pathway, several genetic alterations are known to be associated with androgen independence. A correlation between an increase in HER-2/*neu* expression and progression to androgen independence has been shown recently (7). In addition, when assessed in a mouse model as a single agent, trastuzumab (Herceptin), an antibody generated against the extracellular domain of Her-2/*neu*, showed activity in the androgen-dependent tumor tested. An additive effect on growth was also detected in com-

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³ The abbreviations used are: AIPC, androgen-independent prostate cancer; p21, p21^(WAF-1/CIP1); IL, interleukin.

Table 1 Clinical characteristics of 105 patients with prostate cancer^a

Patients (no.)	Clinical stage	Androgen status	Previous therapy	Specimen
Group I				
3	A	AD	None	Prostate
11	B	AD	None	Prostate
6	C	AD	None	Prostate
Group IIA				
14	D1	AD (9), AI (5)	Hormone (6), RX (1), chemo (1), none (7)	Prostate (6), LN (8)
13	D2	AD (1), AI (12)	Hormone (12), RX (8), chemo (7), none (4)	Prostate (6), LN (4), bone (1), brain (1), bladder (1)
Group IIB				
58	D2	AI (58)	Hormone (58), chemo (12), RX (21), strontium 89 (2)	Bone marrow (58)

^a AD, androgen dependent; AI, androgen independent; LN, lymph nodes; Hormone, androgen ablation; RX, radiotherapy or brachytherapy; chemo, chemotherapy.

bination with paclitaxel in both androgen-dependent and androgen-independent tumors (8). However, preliminary results from a clinical trial using trastuzumab suggested no efficacy or limited efficacy in AIPC (9), in contrast to what was shown in breast cancer (10). Accumulation of the p53 tumor suppressor protein (a reliable indicator of p53 gene mutations in prostate cancer) correlates with advanced disease and progression toward androgen independence (11, 12). Similarly, progression to androgen independence is associated with an increased proportion of cells that express *bcl-2*, an antiapoptotic gene (13–15). Direct evidence of a facilitating effect of *bcl-2* on the multistep prostate carcinogenesis has been established recently (16), and antisense *bcl-2* oligodeoxynucleotides delayed androgen independence relapse (17). Interestingly, p53 accumulation and *bcl-2* expression are independent events in AIPC (14). Moreover, abnormalities of either one or both of these genes are detected in only roughly two-thirds of AIPCs, suggesting the existence of at least one other genetic alternative pathway.

The *p21* gene has been identified as a key factor for the regulation of cell growth. p21 protein expression induces G₁ arrest by inhibiting the activity of the cyclin-dependent kinases and interacting with the proliferation of cell nuclear antigen, thereby directly preventing DNA synthesis (18, 19). Whereas the binding of p21 inhibits replication, it does not affect the repair activity of proliferation cell nuclear antigen *in vitro*. Recent *in vitro* data suggested that p21 modulates p53-induced apoptosis, as well as prostate cancer cell survival, after exposure to DNA-damaging agents and growth factor deprivation (20). In the present study and for the first time to our knowledge, we have analyzed the patterns of p21, p53, *bcl-2*, and androgen receptor expression in tumor samples from patients with prostate cancer, including 58 bone metastasis from AIPC. An association between p21 expression and the progression of prostate cancer to androgen independence is strongly suggested by our results and is also supported by our data obtained in a mouse model of AIPC.

MATERIALS AND METHODS

Patient Population. Patient medical records were obtained from the departments of Genitourinary Medical Oncology and Urology at The University of Texas M. D. Anderson

Cancer Center. Androgen status was determined for each patient. All patients had their disease clinically staged according to the modified Whitmore-Jewett staging system (21). Androgen-independent cancer was defined as tumors from patients whose disease showed no initial response to androgen deprivation or who experienced disease progression after initial disease response. An increase in prostate-specific antigen level, a tumor bulk increase of a measurable lesion, or the development of additional metastases in the presence of castrate levels of serum testosterone were considered evidence of disease progression. The study population included 20 patients with organ-confined or locally advanced prostate cancer (group I), 27 patients with early (stage D1) and late (stage D2) disseminated prostate cancer (group IIA), and 58 patients with bone metastasis from AIPC from whom a bone marrow biopsy specimen of a bone metastasis had been obtained (group IIB). This classification was designed to provide two homogeneous groups of patients (groups I and IIB) reflecting different anatomical and biological stages of the disease. Table 1 summarizes the clinical features of these patients. All patients had histologically proven adenocarcinoma of the prostate.

Tissue Samples. Formalin-fixed, paraffin-embedded tumor samples from a total of 105 patients were used in this study. The prostate cancer specimens were obtained from the pathology files of the M. D. Anderson Cancer Center's Division of Pathology. Primary prostate cancer samples from patients in group I were obtained by radical prostatectomy (*n* = 10), cystoprostatectomy (incidental prostate cancers, *n* = 3), biopsy (*n* = 5), and transurethral resection of the prostate (*n* = 2). Samples from patients in group IIA were obtained by biopsy of the prostate, metastatic sites, retroperitoneal lymph node dissection, or decompression laminectomy. Samples from patients in group IIB consisted of a bone marrow biopsy specimen in all cases. Bone marrow biopsy specimens were decalcified in 5% formic acid. Serial 3- μ m tissue sections were cut from each sample; one section was stained with H&E, and adjacent sections were used for immunostaining as detailed below. Table 1 summarizes therapy received by patients before tissue specimen procurement.

Cell Lines. MDA PCa 2b cells (22, 23) were propagated in BRFF-HPC1 (Biological Research Faculty and Facility, Inc.,

Jamesville, MD) with 20% fetal bovine serum (Sigma Chemical Co., St. Louis, MO) and Gentamicin (50 µg/ml; Life Technologies, Inc., Gaithersburg, MD).

In Vivo Study. Six- to 8-week-old male athymic (nude) mice (Charles River Laboratory, Wilmington, MA) were used for the tumorigenicity assay. The mice were housed under constant humidity and temperature, with 12-h light/12-h dark cycles. The mice were allowed *ad libitum* access to standard mouse feed and water and were monitored daily. Mice were injected with 4×10^6 MDA PCa 2b cells s.c. in the right axilla area. Tumor development in each animal was followed by caliper measurements twice weekly (tumor volume was calculated as length \times width \times height \times 0.5236, the formula of an ellipsoid).

Surgical castration was performed as described previously (22). After sacrifice, the collected tumor samples were fixed in 4% paraformaldehyde in PBS and embedded in paraffin, and serial 3-µm tissue sections were cut from each sample. One section was stained with H&E, and adjacent sections were used for immunostaining as described below.

Immunohistochemical Analysis. Immunohistochemical studies were performed on both formalin-fixed, paraffin-embedded tissues sections of the human prostate carcinoma specimens and MDA PCa 2b-induced tumors in nude mice using the streptavidin-biotin-peroxidase method (24, 25). Sections were cut 3-4-µm thick, deparaffinized in xylene, and rehydrated in descending grades (100-70%) of ethanol. Endogenous peroxidase activity was blocked by a 10-min treatment with 3% hydrogen peroxide in absolute methanol. To enhance the immunostaining, a heat-induced epitope retrieval procedure was performed using a Black and Decker vegetable steamer (Gelton, CT). Briefly, deparaffinized sections were placed in a thermo-resistant container filled with citrate buffer solution (pH 6.0), steamed for 45 min, and then cooled for 20 min.

Sections were then incubated with a primary antibody in a humid chamber for 1 h at room temperature. The primary antibodies used were monoclonal antibodies to p53 (DO-7; DAKO Corp., Carpinteria CA, 1:100 dilution), Ki-67 (MIB1; DAKO Corp., 1:125 dilution), p21 (Ab-1; Oncogene Research Products, Cambridge, MA, 1:40 dilution), Bcl-2 (clone 100; BioGenex, San Ramon, CA, 1:200 dilution), and androgen receptors (F39.4, 1:100 dilution). This was followed by immunoperoxidase staining using the LSAB2 peroxidase kit (DAKO). The immunostaining reaction was visualized using 3,3'-diaminobenzidine as chromogen. Slides were counterstained with Mayer's hematoxylin and mounted with Permount. To evaluate the specificity of the antibodies, known positive and negative tissues were used as controls.

p53, bcl-2, and androgen receptor expression was considered positive if >5% of tumor cells were stained throughout the tissue section. p21 expression was considered positive if >5% of tumor nuclei were stained throughout the tissue section or >20% of tumor nuclei were stained in a single microscopic field. Cases with no or only single positive cells were regarded as negative. The immunoreaction of Ki-67 was assessed in the samples, and the percentage of positive nuclei was defined as the Ki-67 index. A Ki-67 index was classified as high if it was >20%. This cutoff point was based on previous published

Table 2 p21 expression in prostate cancer

Tumor characteristics	
Cases with positive p21 staining	
Group I: localized PC ^a	5/20 (25%)
Group IIB: disseminated AIPC, bone disease	27/58 (47%)

^a PC, prostate cancer.

studies correlating high Ki-67 proliferating index to worse clinicopathological parameters (24, 26).

Statistical Analysis. Percentages were compared using the χ^2 test and Fisher's exact test.

RESULTS

Correlation of p21 Expression with Androgen Independence and Stage in Prostate Cancer. The expression of p21 was determined in 105 patients with various stages of prostate cancer (Table 2). p21-positive immunostaining was detected in the nuclei of prostate cancer cells in 5 of 20 (25%) patients with localized disease and in 27 of 58 (47%) patients with disseminated disease of whom we had a bone marrow biopsy (Table 2). Fig. 1, A and B illustrates prostate cancer specimens (primary and bone metastasis) with p21-positive immunostaining; Fig. 1C shows a prostate cancer specimen scored as negative. No detectable p21 staining was detected in the normal prostate (Fig. 1D). There was an association between p21 expression and androgen-independent status, because 7 of 30 (23%) androgen-dependent tumors and 36 of 75 (48%) androgen-independent tumors stained positively for p21 ($P < 0.02$). There was no significant correlation between p21 expression and a high Gleason score in patients with localized prostate cancer, although the limited number of cases does not allow definitive conclusions. To assess the prognostic role of p21 in patients with AIPC and bone disease, survival rates of patients from group IIB were studied. p21 had no prognostic value when studied in this homogeneous subgroup (data not shown).

p21 Expression Correlates with Tumor Proliferation in Bone Metastasis from AIPC. Samples from bone metastases from AIPC (group IIB) were stained for Ki-67 as an indicator of cell proliferation. Ki-67 identifies cells that are cycling because it reacts with a nuclear antigen that is present in the G₁, S, G₂, and M phases of the cell cycle but not in the G₀ phase (27). Among these tumors, 22 of 55 (40%) had a high (>20%) Ki-67 index. There was a significant correlation between p21 expression and a high Ki-67 index: 14 of 26 (54%) p21-positive tumors and 8 of 29 (28%) p21-negative tumors had a high Ki-67 index ($P < 0.05$). Moreover, a comparison between p21-stained and Ki-67-stained slides showed that the expression of both proteins overlapped (data not shown). These results suggest that p21 expression is associated with cell proliferation.

p21 Expression Occurs Independently of p53, bcl-2, and the Androgen-receptor Expression in Bone Metastasis from AIPC. Because accumulation of p53 and expression of bcl-2 have been shown to be the most frequent molecular abnormalities seen in AIPC besides those involving the androgen receptor pathway, a subset of tumors from group IIB with sufficient tissue was studied by immunohistochemistry for expression of

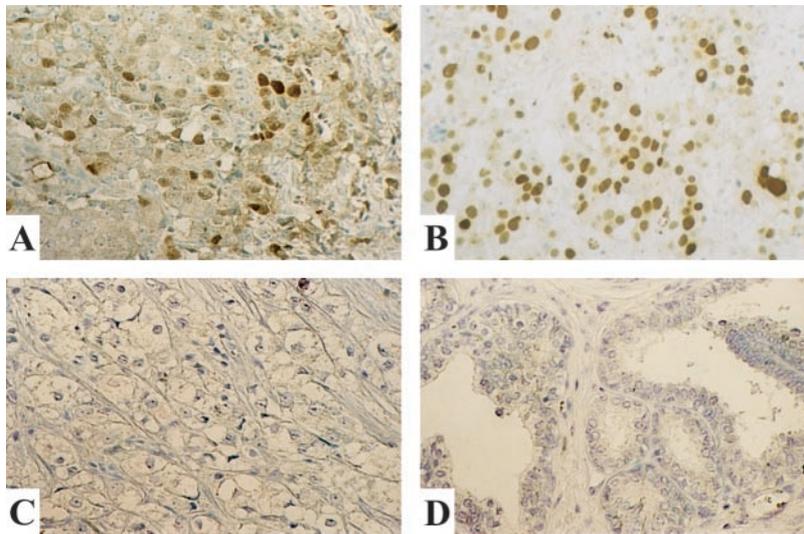


Fig. 1 Photomicrographs of tissue sections of prostate cancer immunostained with antibody to p21 ($\times 250$). A, primary prostate cancer positive for p21 expression. B, bone metastases of prostate cancer positive for p21 expression. C, primary prostate cancer negative for p21 expression. D, normal prostate negative for p21 immunostaining.

Table 3 Expression of p53, p21, bcl-2, and the androgen receptor in bone metastases from AIPC

Protein expression	No. of positive cases (%)
p53	27/43 (63%)
p21	20/43 (47%)
bcl-2	14/43 (33%)
Androgen receptor	34/35 (97%)

p53, bcl-2, and the androgen receptor. Data were correlated to p21 staining. Results are summarized in Table 3. p53 protein accumulation occurs in 63% of cases. No correlation was found between p53 and p21 expression ($P = 0.12$). One-third of AIPC cases expressed bcl-2, and no correlation was found with p21 expression ($P = 0.74$). Interestingly, 4 of 43 (9%) AIPC cases expressed p21 and had negative staining for p53 and bcl-2. Positive staining for the androgen receptor was found in 34 of 35 (97%) samples. The staining consisted of a high proportion of positive cells in most of the cases: $>70\%$ of the sample ($n = 29$), 30–70% of the sample ($n = 3$), and $<30\%$ of the sample ($n = 2$). There was no evidence of an association between p21 expression and the proportion of cells that stained positive for the androgen receptor protein (three of the five cases with $<70\%$ positive cells for the androgen receptor were positive for p21).

p21, p53, and Ki-67 Expression after Castration in an *In Vivo* Model of Prostate Cancer. Our clinical data suggested a correlation between p21 expression and the progression to the androgen-independent status. However, it was difficult to ascertain whether this was not only an apparent association, because there was also a trend between p21 expression and disease stage (Table 2). We could not rule out this hypothesis using our clinical specimens, because all patients with localized prostate cancer had androgen-dependent disease, whereas our patients with bone metastases had androgen-independent disease. Therefore, we decided to verify this apparent association in an *in vivo* xenograft model of prostate cancer.

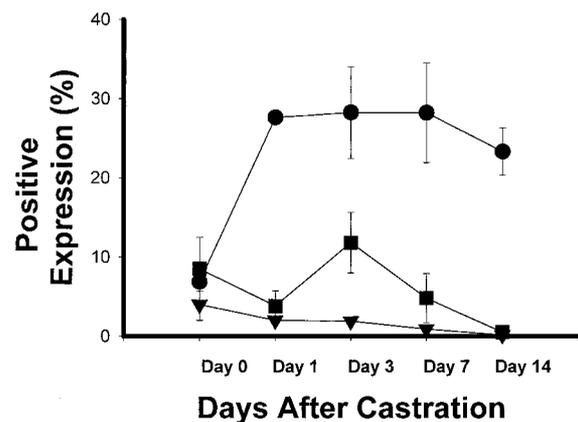


Fig. 2 Expression of p21, p53, and Ki-67 assessed by immunohistochemistry in MDA PCa 2b tumors after castration of nude mice. Surgical castration was performed when the tumor had reached 300 cm^3 . Then, five mice were selected randomly and killed at each time point. ●, p53; ■, p21; ▼, Ki-67.

Twenty-five 6–8-week-old male nude mice were injected with 4×10^6 MDA PCa 2b cells s.c. Surgical castration was performed when the tumor volume had reached 300 mm^3 . In this model, the xenograft tumor stopped growing 2–3 weeks after castration and decreased in size in most cases. In $\sim 40\%$ of cases, the tumor grew again, thus defining androgen independence. Five mice were randomly killed at each time point (days 0, 1, 3, 7, and 14), and the expression of p21, p53, and Ki-67 was assessed on the resected tumors by immunohistochemistry. The results are shown in Fig. 2 and indicate that the expression of p21 decreases after castration and is almost no longer detectable 2 weeks after castration. These results were confirmed in a separate experiment using a simplified time course (days 0 and 15 only). Similar results were also obtained when the MDA PCa 2a cell line was used in place of the MDA PCa 2b cell line (data not shown). The Ki-67 index also rapidly decreased after castration. In contrast, p53 staining increased from day 1 after

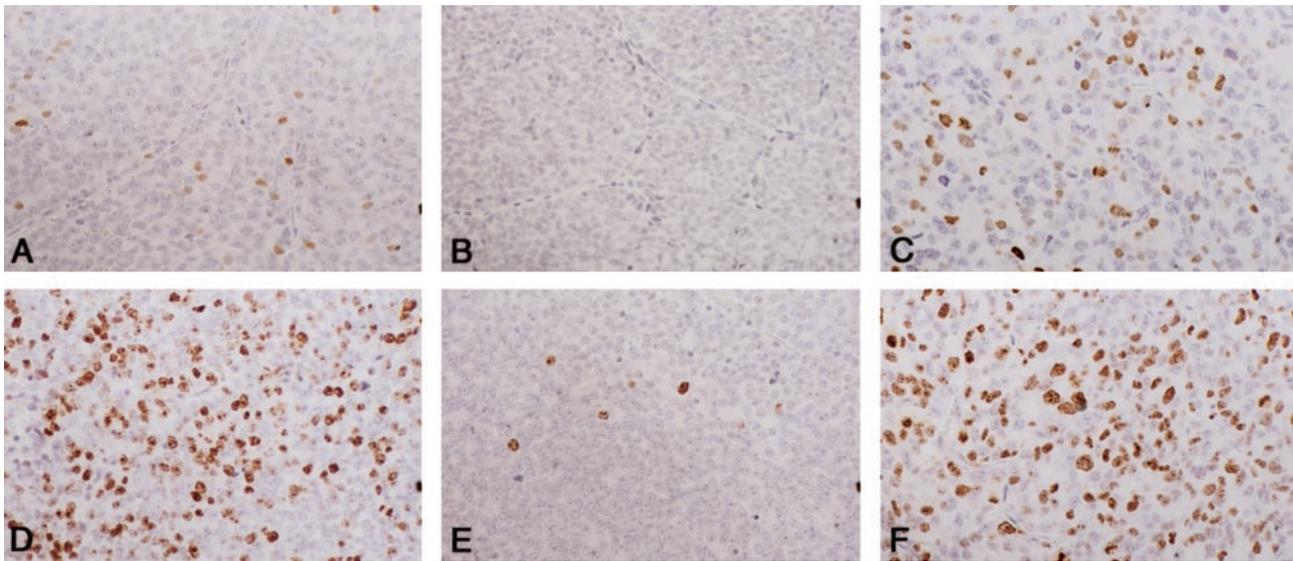


Fig. 3 Expression of p21 assessed by immunohistochemistry in MDA PCa 2b tumors grown in nude mice before castration (A), 4 weeks after castration (B), and at relapse (C). Expression of Ki-67 in the same areas of MDA PCa 2b tumors grown in nude mice before castration (D), 4 weeks after castration (E), and at relapse (F).

castration and remained elevated during the time course (Fig. 2). Although castration results in decreased tumor size in this model, there was no evidence of increased apoptosis as assessed by terminal deoxynucleotidyl transferase-mediated nick end labeling staining (data not shown). These results suggest that the balance between proliferation and apoptosis will determine tumor size in this model. Similar findings were reported by other groups (28).

Increased p21 Expression at Androgen-independent Relapse. A group of 9 male nude mice were injected with 4×10^6 MDA PCa 2b cells s.c. and subjected to surgical castration as described. Tumor bulk was measured every week. When tumor bulk increased again for ≥ 2 consecutive weeks, it was considered that the tumor had become androgen independent. The mouse was then killed, and the tumor was subjected to p21 and Ki-67 immunostaining. Four mice relapsed, of whom three were available for pathological studies. All three tumors stained positive for p21 in the following proportions: 40, 10, and 10% (Fig. 3). Moreover, the positive areas showed a particular phenotype with a lower cell density compared with the negative areas (Fig. 3C). There was no correlation between areas staining positive for p21 and areas staining positive for p53 (data not shown). In contrast, there was a correlation between areas staining positive for p21 and those staining positive for Ki-67. Moreover, p21-positive areas were those which exhibited high mitotic activity.

DISCUSSION

We show here that p21 expression is associated with cell proliferation and androgen-independent disease in patients with prostate cancer. The association of p21 with androgen independence was confirmed in an *in vivo* model of AIPC.

Previous reports have shown that about one-fourth to one-third of localized prostate cancer expresses p21 (25, 26, 29) and

that the differences in positive rates are probably related to the different criteria for prostatectomy among the treating physicians. Our study's positive rate of 25% is in this range. The proportion of positive cells among the tumors is typically low, in the 10% range (25, 30, 31), and we confirmed this finding. Although p21's prognostic role is debated (30, 31), some reports have shown that it has an unfavorable impact in patients with localized prostate cancer, because its expression is positively correlated with the risk of prostate-specific antigen relapse (26, 29).

The most important new finding of the present study is that p21 expression is associated with progression toward androgen-independent disease. The proportion of p21-positive tumors in our set of patients with AIPC was 48%. *In vivo*, p21 and Ki-67 expression rapidly decreased after castration to become almost undetectable after 14 days. This pattern is in accordance with what was reported previously by others using the CWR22 mouse model (28). However, in this latter model, the drop in p21 expression paralleled a p53 expression decrease. In contrast, in our model, p21 decreased, whereas p53 remained at a high level of expression. This might be because p21 can also be activated or inactivated through a p53-independent pathway to regulate the cell cycle (32, 33). Relapse, defining the androgen-independent status, was associated with an increase in p21 expression, which reaches an even higher level than seen before castration. This finding and the fact that p21 is associated with poor outcome in patients with prostate cancer can be regarded as unexpected events, because it is usually acknowledged that p21 is a growth-inhibitory molecule. Obviously, the accumulated p21 in the androgen-independent cases reported here and in poorly differentiated prostate cancer cells reported by others (29) is not able to inhibit tumor growth. This phenomenon is unlikely to be related to genetic alteration of the *p21* gene, because mutations and polymorphism of this gene are rare in

human neoplasms, including prostate cancer (34, 35). Another more plausible explanation involves the recent discovery that p21-induced cells may have a paracrine growth-stimulatory effect. Indeed, Chang *et al.* (36) showed evidence that the conditioned media from p21-induced cells has antiapoptotic and mitogenic properties on noninduced cells, although the overexpression of p21 induces growth arrest *in vitro*. The molecular events underlying this effect may involve activin A, epithelin/granulin, and galectin-3 mitogenic proteins and the prosaposin apoptotic inhibitor, because the genes of these soluble factors are activated in cells expressing p21 constitutively. If these *in vitro* events also occur *in vivo*, this might clearly be an explanation for the apparent association between p21 expression and AIPC.

Another possible explanation involves the regulation of p21 expression by the androgen receptor. Indeed, several recent studies showed that androgen up-regulates expression of p21 and suggest that p21 may have an antiapoptotic function in prostate cancer (37–39).

Our study shows that the androgen receptor is expressed in most (97%) bone metastasis samples from AIPC. These results confirm previous studies based on biopsies of the primary prostate cancer, lymph node metastasis, or a limited number of bone metastases (40–42). We did not find any association between p21 expression and the level of expression of the androgen receptor in bone metastases. However, if it exists, this association would be difficult to detect because of the high expression level of the androgen receptor (>70% positive cells in 83% of the cases in our study). Finally, IL-6, a cytokine with growth-promoting activity in different cell types, has been implicated in the progression of prostate cancer and associated with the metastatic phenotype. IL-6 has been shown to mediate ligand-independent activation of the androgen receptor pathway in prostate cancer cells (43, 44) and up-regulation of p21 expression in melanoma, osteosarcoma, and myeloid cells (45–47). IL-6 may, therefore, modulate p21 expression through androgen-dependent or -independent pathways in AIPC.

Because only roughly 50 and 30% of AIPC cases are positive for p53 and bcl-2, respectively (11–15), it is likely that other major molecular events occur to allow cells to grow in an androgen-depleted environment. In AIPC, an association between bcl-2 and p21 expression has been suggested recently (48) but was not confirmed in the present study. Interestingly, p21 staining was not always associated with a p53-positive or bcl-2-positive staining in our series of patients with AIPC. Indeed, 4 of 43 (9%) cases display a p53⁻/bcl-2⁻/p21⁺ phenotype. Therefore, these results raise the possibility that p21 expression favors the progression of a subpopulation of prostate cancers to androgen-independent status. This finding may be of relevance for therapeutic targeting, because the antisense strategy directed to p21 has proven effective *in vitro* (20), whereas gene therapy using wild-type p53 is under development in the prostate cancer setting at The University of Texas M. D. Anderson Cancer Center (49, 50).

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