Loss of the Cyclin-dependent Kinase Inhibitor p27\textsuperscript{Kip1} Protein in Human Prostate Cancer Correlates with Tumor Grade\textsuperscript{1}

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ABSTRACT

Loss of expression or mutational deletion of the cyclin-dependent kinase inhibitor p27\textsuperscript{Kip1} has recently been implicated in malignant development. In this study, we investigated the relationship between p27\textsuperscript{Kip1} protein expression and tumor grade in human prostate cancer by conducting an immunohistochemical analysis in a series of normal prostate, benign prostatic hyperplasia, and malignant prostate cancer specimens. The proliferative activity of prostatic tumors was determined on the basis of the Ki-67 nuclear antigen staining. A uniformly intense immunoreactivity for p27\textsuperscript{Kip1} was localized to the nuclei of glandular epithelial cells of normal prostates. The benign glandular epithelia exhibited moderate immunostaining. In the malignant prostate tissue, however, a heterogeneous pattern of substantially reduced p27\textsuperscript{Kip1} immunoreactivity was found among the glandular epithelial cells. The majority of primary prostate cancer specimens (68%) were totally negative for p27\textsuperscript{Kip1} immunoreactivity, whereas the rest exhibited a significantly decreased p27\textsuperscript{Kip1} expression, compared with the normal prostate (P < 0.01). Moreover, there was progressively diminished p27\textsuperscript{Kip1} immunostaining with increased tumor grade. This loss of p27\textsuperscript{Kip1} was associated with an increase in the proliferative index of prostatic tumors (r = 0.88). There was no significant relationship between p27\textsuperscript{Kip1} loss and the transforming growth factor β receptor status of prostatic adenocarcinomas. These results indicate that frequent loss of the cyclin-dependent kinase inhibitor p27\textsuperscript{Kip1} in human prostate cancer cells correlates with advancing histological aggressiveness, implicating deregulation of p27\textsuperscript{Kip1} in prostate tumor progression.

INTRODUCTION

Prostate cancer is the most common malignancy and the second leading cause of cancer mortality in American males (1). In the normal prostate, androgens exert their growth-regulatory mechanisms via growth factor-signaling pathways. Androgen-independent clones of malignant prostate cells, however, may maintain constant growth rates via autocrine production of growth-stimulatory factors or reduced expression or responsiveness to growth-inhibitory factors (2). Identification of factors predictive of the aggressive growth of the malignant prostate would lead to the development of therapeutic interventions to reduce tumor progression and to improve patient survival. Despite intense efforts over the last several years, very few molecular/cellular markers have added significantly to the prognostic information provided by stage and grade.

Studies on the tissue kinetics of human prostate cancer that determined the rates of cell proliferation and cell death (apoptosis) revealed a net increase in the cell number of prostatic tumor epithelial cells due to loss of apoptotic cell death control in favor of cell proliferation (3, 4). A general understanding of how cell proliferation and cell death are regulated by extracellular signals requires the identification of mechanisms that induce cell cycle arrest and apoptosis. TGF-β1 is a multifunctional growth factor that plays a pivotal role in maintaining tissue homeostasis by regulating cell proliferation, cell differentiation, and cell death in several cell types (5). As a physiological regulator of prostate epithelial cell growth, TGF-β acts by arresting actively proliferating cells and inducing apoptosis in both normal and malignant prostate cells (6, 7). TGF-β1 elicits its effects through interactions with the transmembrane receptors, R-I and R-II, which interact with each other to form a heteroduplex. The intracellular domains of these receptors, activated by the binding of the ligand, control the recruitment and activation of intracellular mediators of the TGF-β-signaling pathways (8).

The intracellular events leading to TGF-β-negative growth effects have not been fully elucidated, but they appear to include induction of inhibitors of cyclin-cdk complexes, such as p27\textsuperscript{Kip1}, p21\textsuperscript{WAF1/CIP1}, and p15\textsuperscript{INK4A} (9–13). Mechanistically, p27\textsuperscript{Kip1} protein links TGF-β to cell cycle arrest by being released from cyclin D1-Cdk4 complexes upon TGF-β treatment of TGF-β-sensitive cells to be associated with cyclin E-cdk2 complexes, thus blocking its kinase activity (12–14). Loss of the negative growth regulation by TGF-β, due to deregulation of its expression, or loss of sensitivity to its effect, is believed to contribute to tumorigenic growth. Increasing evidence points to the potential involvement of a dysfunctional TGF-β signaling.

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\textsuperscript{3} The abbreviations used are: TGF-β, transforming growth factor β; R-I and R-II, receptor types I and II, respectively; cdk, cyclin-dependent kinase; BPH, benign prostatic hyperplasia.
pathway in prostate tumorigenesis. Recent studies from this laboratory, as well as by other investigators, have demonstrated a marked decrease in the expression of TGF-β receptors in prostatic tumors, a loss that correlates with tumor grade (15–17). However, prostatic tumors with functional TGF-β receptors may harbor downstream defects, which impair the intracellular transduction of TGF-β signal. As growing evidence implicates the involvement of cdk inhibitors as postreceptor effectors transducing the TGF-β signal (11–13), these molecules may represent critical intracellular targets for the negative growth effects of TGF-β in the human prostate.

In view of the heavily supported notion that p27Kip1 plays a central role in the basic machinery controlling TGF-β-mediated negative growth effects (13, 14), we hypothesized that potential defects in this downstream effector may be involved in abrogating TGF-β signal transduction during prostate carcinogenesis. In this study, we conducted an immunohistochemical analysis to examine the expression and topological distribution of the cdk inhibitor p27Kip1 protein in normal prostate, BPH, and malignant prostate cells. We found that normal and benign cells of the prostatic glandular epithelium express high levels of p27Kip1 protein, whereas in prostate cancer, p27Kip1 immunoreactivity was significantly reduced.

MATERIALS AND METHODS

Prostate Tissue Specimens. Paraffin-embedded sections (6 μm) of human prostate tissue were obtained from the archives of the Department of Pathology at the University of Maryland Medical System. These specimens included 40 primary prostate adenocarcinomas from patients undergoing radical prostatectomy for localized disease (acquired between 1992 and 1995), 30 prostate specimens from patients with BPH, and 5 lymph nodes positive for metastatic deposits of prostatic tumor cells from patients who had undergone laparoscopic pelvic lymph node dissection. Normal prostate tissue was obtained from six age-matched men undergoing cystoprostatectomy (for bladder cancer). The tumor histological grade of all of the prostatic adenocarcinomas was evaluated by the pathologist (A. B.), using the Gleason scoring system (18), prior to immunohistochemical analysis.

Immunohistochemical Analysis. Tissue sections were deparaffinized and dehydrated with xylene and graded ethanol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol at room temperature for 20 min. After washing with PBS (three times, 5 min each), nonspecific binding sites were blocked with 10% goat serum (45 min). Slides were subsequently incubated with an antibody against the human p27Kip1 protein (1 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. The antibody against the p27Kip1 protein was a rabbit polyclonal antibody raised against the peptide sequence (amino acids 181–198 mapping at the COOH terminus of human p27Kip1). After PBS rinsing, sections were incubated with the biotinylated antirabbit secondary antibody and were subsequently treated with the biotin-avidin peroxidase system (Santa Cruz Biotechnology). Positive immunoreactivity was detected by the diaminobenzidine reaction. Hematoxylin was used for counterstaining. Immunohistochemical analysis of TGF-β R-I and R-II expression was performed using antibodies from Santa Cruz Biotechnology, as described previously (17). Quantitation of the immunohistochemical staining of the sections was performed by two independent observers (Y. G. and N. K.) under the microscope. Cell counts were performed at ×400 magnification with a 10 × 10 grid in the eyepiece, with at least 500 cells in four different fields counted, and mean percentage of immunoreactivity was expressed as the percentage of the number of positively stained cells divided by the total number of cells. To evaluate the relative staining intensity, immunoreactivity was subjectively graded 0, +1, +2, +3, indicating negative, weak, medium, and strong staining, respectively. The proliferative index of prostatic tumor cell populations within the same sections was determined on the basis of immunostaining for Ki-67 antigen, using the mouse monoclonal antibody M1B from AMAC, Inc. (Westbrook, ME), as described previously (4). Cells showing any nuclear staining were considered to be Ki-67 positive. The proliferative index was determined by examining areas with homogeneous distribution of tumor cells within the section. Counting was based on the distribution of Ki-67-positive cells in four different fields within the same section and expressed as a percentage of the total number of cells. Control sections in which the primary antibody was omitted (substituted with nonspecific rabbit IgG) revealed a total lack of immunoreactivity.

Statistical Analysis. The Wilcoxon rank sum test for nonparametric methods was used to analyze the differences in p27Kip1 immunoreactivity between normal prostate, BPH, and prostate cancer of Gleason grades 2, 3, 4, and 5 (19). Mean values were considered significant at a P of 0.01. The correlation coefficient between the p27Kip1 loss and the TGF-β R-II loss, as well as the proliferative index of prostatic tumors, was determined by linear regression analysis (19).

RESULTS

In normal prostatic epithelial cells (cystoprostatectomy specimens), the level and intensity of p27Kip1 immunostaining was uniformly high (+3; Fig. 1, A and B). Immunoreactivity was concentrated within the nucleus toward the nuclear membrane, but diffuse cytoplasmic staining was also observed. Benign cells of the prostatic glandular epithelium in BPH specimens expressed moderate levels of p27Kip1 (+2 intensity), whereas stromal staining was rather diffuse and of weak nature (Fig. 1C). In marked contrast, p27Kip1 immunoreactivity was consistently lower in prostate cancer compared to the normal and benign prostatic glandular epithelia (Fig. 1D). Fig. 1D reveals a well-differentiated prostate adenocarcinoma stained with p27Kip1 antibody, exhibiting total loss of p27Kip1 immunoreactivity. The results summarized in Table 1 reveal the distribution of immunostaining for p27Kip1 among the various prostate tissue types. Approximately 68% of the primary adenocarcinoma specimens included in this study exhibited total loss of p27Kip1 staining. Interestingly, although the majority of BPH sections analyzed exhibited moderate to weak p27Kip1 immunoreactivity, carcinoma foci adjacent to benign tissue (within the same sections BPH sections) were negative for p27Kip1 immunostaining (Table 1). Two of the five lymph nodes, positive for metastatic deposits of prostate cancer (40%),
totally lacked p27Kip1 expression, whereas the other three exhibited weak to medium immunoreactivity.

A moderate degree of variability in the staining intensity was observed among cancer foci of the same histological grade. Moderate immunoreactivity for p27Kip1 was detected in 8 of the 40 primary tumors analyzed (20%), and this was only present in focal areas of the tumors. Table 2 indicates a summary of the quantitative analysis of p27Kip1 immunoreactivity related to the histological grade of prostatic tumors. These results indicate a significant decrease in the amount of p27Kip1 immunoreactivity with increasing Gleason score of the prostate adenocarcinomas (P < 0.01). All Gleason 5 cancer foci were totally negative for p27Kip1 staining.

Considering the strong in vitro evidence that established p27Kip1 as a faithful intracellular effector of the TGF-β signal transduction (10–12, 14), it was intriguing to investigate whether those tumors with loss of and/or reduced p27Kip1 expression also have deregulated expression of TGF-β receptors. We thus subsequently examined the relationship between the TGF-β receptor status and p27Kip1 immunoreactivity in 21 of the 40 primary prostate cancer specimens (analyzed for R-I and R-II staining). No significant correlation was detected between loss of p27Kip1 protein and loss of TGF-β R-I or R-II expression (data not shown).

The Ki-67 antibody recognizes a nonhistone protein expressed throughout the cell cycle but absent in the G0 phase. To

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**Table 1** Expression of p27Kip1 protein in normal and malignant human prostate

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>Number of specimens</th>
<th>Distribution of p27Kip1 staining intensity (% of group with)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal prostate</td>
<td>6</td>
<td>0 0 0 100</td>
</tr>
<tr>
<td>BPH</td>
<td>30</td>
<td>3.3 33.4 60 3.3</td>
</tr>
<tr>
<td>Prostate cancer</td>
<td>40</td>
<td>67.5 12.5 20 0</td>
</tr>
<tr>
<td>Cancer in BPH tissue</td>
<td>5</td>
<td>80 20 0 0</td>
</tr>
<tr>
<td>Metastatic prostate cancer</td>
<td>5</td>
<td>40 40 0 0</td>
</tr>
</tbody>
</table>

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**Fig. 1** Photomicrographs of immunoreactivity patterns of p27Kip1 expression in normal prostate, BPH, and malignant human prostatic tissue. Using the rabbit polyclonal antibody against p27Kip1 revealed abundant nuclear immunostaining among the epithelial cells of the prostatic glandular epithelium in the normal prostate. Magnification: ×50 (A) and ×100 (B). C, a section of BPH tissue indicating moderate immunoreactivity of the p27Kip1 protein among the benign prostatic epithelia (+2). D, a primary prostate adenocarcinoma tissue section stained with p27Kip1 antibody, exhibiting loss of p27Kip1 protein expression. The Gleason grade of these cancer foci was 4. Magnification: ×100 (C and D).
Table 2  Correlation of p27Kip1 expression with Gleason grade in primary prostatic tumors

Values represent the mean percentage of p27Kip1-immunoreactive cells (+1, +2, or +3 staining intensity) divided by the total number of cells counted ± SE. Numbers in parentheses indicate the number of specimens analyzed for each group.

<table>
<thead>
<tr>
<th>Tumor grade</th>
<th>Normal (6)</th>
<th>Gleason 2 (29)</th>
<th>Gleason 3 (33)</th>
<th>Gleason 4 (10)</th>
<th>Gleason 5 (4)</th>
</tr>
</thead>
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<tr>
<td>% p27Kip1 positivity</td>
<td>79.3 ± 16.8</td>
<td>38.8 ± 14.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.5 ± 12.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.4 ± 2.6&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
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<sup>a</sup> Values significantly different from the normal prostate (P < 0.01).

Fig. 2  Relationship between loss of p27Kip1 protein expression and the proliferative activity of prostatic tumors. A direct correlation was detected between loss of p27Kip1 immunoreactivity and the proliferative index (as determined by the percentage of Ki-67-positive cells) of prostatic cancer specimens (correlation coefficient, r = 0.88).

elucidate the biological significance of deregulation in p27Kip1 expression in prostate cancer, the proliferative index was determined (on the basis of the percentage of Ki-67-positive cells) in 22 of the primary prostate cancer specimens included in this study. There was an association between the loss of p27Kip1 expression and the proliferative index of primary prostatic tumors (Fig. 2). The increase in the proliferative indices of primary prostatic tumors strongly correlated with the loss of expression of p27Kip1 protein (r = 0.88).

DISCUSSION

The cdk5 lay at the heart of the growth control apparatus via their ability to regulate activation of key players triggering cell cycle transition (10, 20). Recent genetic studies have demonstrated that loss of expression and function of the cdk inhibitor p27Kip1 leads to development of multiple organ hyperplasia and malignancy (21, 22). In this study, we have examined the expression of this cdk inhibitor, p27Kip1, in normal prostate, BPH, and malignant human prostate. A significant loss of immunoreactivity of p27Kip1 protein was detected in primary and metastatic prostate cancer, compared to the normal prostate and BPH. The focal variation in p27Kip1 expression, observed in certain prostatic tumors, may reflect genetic instability of the tumor. Significantly enough, our findings demonstrate that the higher the Gleason grade of prostatic cancers, the greater the likelihood for total loss of immunoreactivity for p27Kip1. This positive correlation implies that with advancing tumor grade, loss of a key cell cycle regulator, p27Kip1, may have prognostic value in human prostate cancer.

Southern blot analysis is currently being conducted to determine whether this loss and/or reduced expression of p27Kip1 protein is due to a reduced gene dosage (and homozygous deletion) or a mutational change (single-strand conformational polymorphism analysis). A recent report suggests that genetic alterations of the p27Kip1 gene are rare in human malignancies, including prostate cancer (23). These findings how-
ever, do not necessarily challenge the potential involvement of p27kip1 protein deregulation in prostate tumorigenesis, because translational (rather than transcriptional) control is primarily responsible for the regulation of p27kip1 during cell cycle progression in response to TGF-β (24). Moreover, a recent study has implicated up-regulation of p27kip1 protein as the mechanism underlying the antiproliferative effect of epidermal growth factor blockade in human prostate cancer cells (25). This evidence, in accord with our findings, points to a potential role for p27kip1 as a critical cell cycle checkpoint in prostate cancer cells.

The present results, indicating deregulation of expression of a TGF-β signal intracellular effector, p27kip1, in prostate cancer cells, may provide an explanation for the TGF-β unresponsiveness characterizing certain prostatic tumors, despite the abundance of the ligand TGF-β (4, 26) and the presence of R-I and R-II (17). The lack of a direct correlation between p27kip1 loss and TGF-β receptor status found in this study suggests that a deregulation in p27kip1 protein levels may be responsible for a defective TGF-β signal transduction and ultimate abrogation of the TGF-β negative growth effects in a subset of prostatic tumors that express functional TGF-β receptors.

One might argue that a reduced detection of p27kip1 immunostaining in prostate cancer could be the result of the p27kip1 protein being associated with cdk-4, an interaction that might induce substrate-bound conformational changes that diminish antibody binding. Because upon release from cdk-4, p27kip1 binds to cyclin E-cdk-2 complex (11, 12), we are currently conducting an immunohistochemical analysis of cyclin E expression in prostatic tumors to determine the relationship between cyclin E immunoreactivity with p27kip1 levels within the same tumor cell populations. The existing contradictory reports on the expression of cyclin E in prostate cancer fail to illuminate this matter: although a recent immunohistochemical study demonstrated that cyclin E is rarely expressed in prostate cancer (27), Keyomarsi et al. (28) reported high levels of cyclin E in a small number of prostatic tumors by immunoblotting analysis.

A trend toward a worse prognosis of prostatic carcinoma with a high Ki-67 index has been firmly established by numerous studies (3, 4, 27, 29, 30), but because Ki-67 antigen expression is elevated throughout the cell cycle, the Ki-67 index provides no information regarding the stage of the cell cycle or the kinetics of the population of cycling cells. Several markers of cell cycle progression, however, taken together, may provide useful information on cell cycle kinetics of prostatic tumor cells, which might have prognostic significance (27, 31). One would expect a parameter that is inversely correlated with the proliferative index, such as p27kip1, to be of potential prognostic value. Prospective studies involving a larger number of patients with advanced disease and screening additional cell cycle regulators are required to examine the potential use of p27kip1 as a prognostic marker in prostate cancer.

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Loss of the cyclin-dependent kinase inhibitor p27(Kip1) protein in human prostate cancer correlates with tumor grade.

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