**Advances in Brief**

**Overexpression of the Cyclin-dependent Kinase Inhibitor p16 Is Associated with Tumor Recurrence in Human Prostate Cancer**

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**Abstract**

The INK4A gene maps to the 9p21 region and was initially described [M. Serrano et al., Nature (Lond.), 366: 704–707, 1993; A. Kamb et al., Science (Washington DC), 264: 436–440, 1994] as encoding a 148-amino-acid protein termed p16. The p16 protein associates exclusively with Cdk4 and Cdk6, inhibiting their complexation with D-type cyclins and the consequent phosphorylation of pRb. This contributes to cell cycle arrest. The purpose of the present study was to evaluate patterns of p16 expression in a well-characterized cohort of prostatic adenocarcinomas while exploring potential associations between alterations of p16 and clinicopathological variables. Normal and malignant tissues from 88 patients with prostate carcinoma were examined. In situ hybridization and immunohistochemistry assays were used to determine the status of the INK4A exon 1α transcripts and levels of p16 protein, respectively. Associations between altered patterns of expression and clinicopathological variables, including pretreatment prostate-specific antigen (PSA) level, Gleason grade, pathological stage, and hormonal status, were evaluated using the Mantel-Haenszel χ2 test. Biochemical (PSA) relapse after surgery was evaluated using the Kaplan-Meier method and the log-rank test. Levels of p16 expression and INK4A exon 1α transcripts in normal prostate and benign hyperplastic tissues were undetectable. However, p16 nuclear overexpression was observed in 38 (43%) prostate carcinomas, whereas the remaining 50 (57%) cases showed undetectable p16 levels. Overexpression of p16 protein was found to correlate with increased INK4A exon 1α transcripts. Moreover, p16 overexpression was associated with a higher pretreatment PSA level (P = 0.018), the use of neoadjuvant androgen ablation (P = 0.001), and a sooner time to PSA relapse after radical prostatectomy (P = 0.002). These data suggest that p16 overexpression is associated with tumor recurrence and a poor clinical course in patients with prostate cancer.

**Introduction**

The INK4A gene maps to the short arm of chromosome 9 (9p21) and was initially described as encoding a protein of M, 15,845, termed p16 (1, 2). The p16 protein forms binary complexes exclusively with Cdk4 and Cdk6, inhibiting their kinase activity and subsequent pRb phosphorylation during the G1 phase of the cell cycle (1, 3). Additional complexity results from the presence of a second INK4A product termed p19ARF (4–6). The p19ARF protein has recently been shown to interact with mdm2 and to block mdm2-induced p53 degradation and trans-activation silencing (7, 8). The two products, p16 and p19ARF, share exons 2 and 3 of the INK4A gene, but have distinct promoters and exon 1 units, exon 1α (p16) and exon 1β (p19ARF). The INK4A gene is mutated in a wide variety of tumor cell lines and certain primary tumors (2, 9–14). In addition, methylation of the 5′ CpG island of the exon 1α promoter region is a frequent mechanism of p16 inactivation in primary tumors (15, 16).

In prostate cancer, the role of INK4A has not been well elucidated, though analyses using microsatellite markers in the vicinity of the INK4A gene have revealed loss of heterozygosity in a subset of primary and metastatic prostate tumors (17). Unlike other primary tumors, INK4A inactivation, through either deletions, mutations, or promoter methylation, seems to be an infrequent event in prostate cancer (17–22). The present study uses immunohistochemical and in situ hybridization assays to examine patterns of p16 expression in a well-characterized cohort of prostate cancer patients treated with radical retropubic prostatectomy. Associations between altered p16 phenotypes and clinicopathological variables were also studied to further define their potential implications in prostate cancer.

**Materials and Methods**

**Patient Characteristics and Tissues.** A cohort of patients with prostatic adenocarcinoma undergoing radical prostatectomy at the Memorial Sloan-Kettering Cancer Center (MSKCC) from 1990–1991 was retrospectively evaluated. A total of 88 patients had adequate clinical follow-up and available pathological materials. The median age at the time of surgery was 65 years (range, 46–74 years). The median follow-up time was 64.5 months (range, 10–94 months). Formalin-fixed, paraffin-embedded prostate tissues were obtained from the De-
partment of Pathology. Representative H&E-stained sections were examined to evaluate the histopathological characteristics of each tissue section.

Clinicopathological parameters examined included pretreatment PSA, \(^3\) and pathological stage (23) and Gleason grade (24), both determined based on the radical prostatectomy specimen. The hormonal status of the patients was also evaluated. A portion of the cohort (34 patients or 39%) was treated with neoadjuvant androgen ablation and were defined as hormone-treated. Patients who did not receive neoadjuvant therapy were defined as hormone naïve. Additionally, biochemical relapse was examined. Relapse was defined as an elevation in the serum PSA level in a patient who had previously demonstrated an undetectable PSA level postprostatectomy. That is, only patients who had an undetectable PSA level after surgery were included in the cohort because this indicated that the surgical resection was complete and the patient was free of disease. Patients who had PSA relapse were classified as treatment failures with tumor recurrence.

**Immunohistochemistry.** An avidin-biotin immunoperoxidase assay was performed on formalin-fixed, paraffin-embedded tissue sections. Deparaffinized sections were treated with 1% \( \text{H}_2\text{O}_2 \) to block endogenous peroxidase activity. Sections were subsequently immersed in boiling 0.01% citric acid (pH 6.0) in a microwave oven for 15 min to enhance antigen retrieval, allowed to cool, and incubated with 10% normal horse serum (Organon Technika Corp, Westchester, PA), to block nonspecific tissue immunoreactivities. A well-characterized antibody to p16 (Ab-1, Oncogene Research Products, Cambridge, MA; 2 \( \mu \text{g/ml} \) final concentration) was then incubated overnight at 4°C. Biotinylated horse antimouse IgG antibodies (Vector Laboratories, Inc., Burlingame, CA; 1:25 final dilution) were used as the secondary reagents. This was followed by avidin-biotin immunoperoxidase complexes (1:25, Vector Laboratories, Inc.) for 30 min. Diaminobenzidine was used as the final stain. Immunoreactivities, assessed in tissue sections from a single representative block in each case, were classified as a continuum of data from undetectable levels (0%) to homogenous staining levels (100%). Data were independently obtained by two observers, with minor inter-observer variability that was resolved by review of the problem cases. Tumors were grouped into two categories defined as follows: (a) Group A (≤5% nuclear immunoreactivity in tumor cells); and (b) Group B (>5% nuclear immunoreactivity in tumor cells).

**In Situ Hybridization.** Primers specific for the exon 1α sequence of the \( \text{INK4A} \) gene were used to create digoxigenin-labeled probes for \( \text{in situ} \) hybridization. Probes were cloned into a PCR-Script recombinant plasmid (Stratagene, La Jolla, CA). Plasmid DNA (1 \( \mu \text{g} \)) was linearized using \( \text{BamHI} \) and \( \text{XhoI} \). Antisense and sense riboprobes were generated from \( \text{in vitro} \) transcription of the linearized DNA using T7 and T3 RNA polymerases, respectively. Transcription was sustained for 2 h at 37°C in 1× transcription buffer (Boehringer Mannheim, Indianapolis, IN), 20 units of RNase inhibitor, 10 mmol/liter each ATP, GTP, and CTP, 6.5 mmol/liter UTP, and 3.5 mmol/liter digoxigenin-UTP. Deparaffinized tissue sections were rinsed in water and PBS for 10 min. The slides were digested with Proteinase K (50 \( \mu \text{g/ml} \) for 18 min at 37°C in PBS and postfixed at 4°C in a freshly prepared solution of 4% paraformaldehyde in PBS for 5 min. Prehybridization was done for 30 min at room temperature in 50% formamide and 2× SSC. The hybridization buffer consisted of 50% deionized formamide (v/v), 10% dextran sulfate (50% stock solution), 2× SSC (20× stock solution), 1% SDS (10% stock solution), and 0.25 mg/ml herring sperm DNA (10 mg/ml).

Hybridization was performed overnight at 45°C applying 10 pmol/liter digoxigenin-labeled riboprobe in 50 μl of hybridization buffer per section under a coverslip. The coverslips were removed and the slides were washed twice in prewarmed 2× SSC for 20 min at 42°C, followed by washes in prewarmed 1× SSC and 0.5× SSC, each at 42°C for 20 min. After these washes, the slides were incubated in normal sheep serum diluted in buffer (pH 7.5) and successively in the same buffer with antidigoxigenin-AP antibody (Boehringer Mannheim) at a dilution of 1:500 for 1 h at room temperature. The visualization was accomplished by nitro-blue tetrazolium 5-bromo-4-chloro-3-indolylphosphate. The slides were counterstained with methyl green and mounted.

\( \text{INK4A} \) exon 1α transcript levels were examined in a subgroup of 21 cases. Consecutive tissue sections were used to analyze p16 protein by immunohistochemistry and \( \text{INK4A} \) exon 1α transcript levels by \( \text{in situ} \) hybridization. As in the immunohistochemical analysis, tumors were grouped into two categories defined by the absence (≤5% tumor cells with cytoplasmic staining) or presence (>5% tumor cells with cytoplasmic staining) of transcripts.

**Statistical Methods.** The statistical analyses of the data from the 88 primary prostate cancer patients were conducted as follows. The response variable, time to PSA relapse, was defined as the time from radical prostatectomy to the time of first detectable PSA measurement. Patients who did not achieve a nonmeasurable PSA after radical prostatectomy were excluded from the analysis. Patients who were still alive at the time of analysis without relapse were censored at the date of last follow-up. The baseline variables examined were PSA measurement at the time of diagnosis, hormone status, Gleason score (hormone naïve patients only), stage of disease, and percent of p16 protein expression.

Associations between p16 expression and different categorical variables (hormone status, tumor grade, tumor stage, and pretreatment PSA levels) were assessed by the Mantel-Haenszel \( \chi^2 \) test. Continuous variables, such as pretreatment PSA, not known to follow a particular distribution were also compared between two or more groups using Wilcoxon nonparametric tests.

The Cox proportional hazards model was used to examine the multivariate relationship between PSA relapse-free time from prostatectomy and the baseline variables listed above. The final model was determined using the “all subsets” procedure in SAS PHREG and the Score criterion (25). As normal and benign tissues showed little to no p16 expression, positive expression was described as >5% nuclear expression. This cutoff was specified \( a \) \( \text{priori} \) and used for the subsequent statistical analysis. Immunohistochemical and \( \text{in situ} \) hybridization studies were completed, analyzed, and recorded blind to clinical information.

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\(^3\) The abbreviation used is: PSA, prostate-specific antigen.
Kaplan-Meier estimates of relapse-free survival stratified by p16 classification were evaluated. The LIFETEST procedure in SAS was used to generate the Kaplan-Meier estimates and the resulting survival curves (25, 26). The log-rank test was used to test the hypothesis of no-survival differences between p16-positive and p16-negative populations.

Results

The normal human prostate displayed undetectable levels of p16 protein and INK4A exon 1α transcripts in ductal and acinar epithelial cells. A lack of p16 immunoreactivity in these cells was observed in hormone-treated and hormone-naive cases. Fibromuscular stroma cells also showed undetectable exon 1α transcript levels. A similar negative pattern of p16 expression was observed on the examination of prostatic tissue affected with benign hyperplasia (Fig. 1).

To determine the frequency and potential clinical implications of p16 alterations in prostate cancer, we analyzed a cohort of 88 primary prostate carcinomas. Two patterns of p16 protein expression were noted. We observed that 50 (57%) of the 88 cases had very low (≤5% nuclear immunoreactivity; 7 cases) or undetectable (43 cases) levels of p16 protein expression (Group A; Fig. 2A). In a subgroup of these cases, we also performed in situ hybridization assays, which revealed that all of the cases had undetectable INK4A exon 1α transcripts (Fig. 2B). However, we noted that 38 (43%) of the 88 cases displayed nuclear staining with anti-p16 specific antibodies (Group B; Fig. 2C). Immunoreactivities in tumor cells were further stratified into three categories: (a) 6–29% nuclear staining (n = 11 cases); (b) 30–59% nuclear staining (n = 15 cases); and (c) 60–100% nuclear staining (n = 12 cases). In a subset of these patients, we also conducted in situ hybridization assays with the INK4A exon 1α-specific probe. All of the cases displaying positive immunoreactivities also displayed moderate-to-high levels of exon 1α transcripts (Fig. 2D).

Table 1 summarizes the associations between p16 phenotypes and clinicopathological variables that were assessed by χ² analyses. Immunohistochemical detection of p16 was not associated with Gleason grade, described as either low (Gleason grade 4–6) or high (Gleason grade 7–10; P = 0.153). Similarly, no association was observed between p16 nuclear expression and pathological stage, defined as organ-confined (T₁, T₂) and non-organ-confined (T₃, T₄, or lymph node+; P = 0.087). However, there was a strong association between p16 nuclear expression and pretreatment PSA levels, based on cutoff points of <4, 4–10, and >10 ng/ml (P = 0.018). A similar result was observed when PSA was assessed as a continuous variable (P = 0.01). In addition, we noted a significant correlation between p16 nuclear expression and the use of neoadjuvant androgen ablation (P = 0.001).

A strong association was also found between p16 nuclear overexpression and tumor recurrence, as defined by biochemical (PSA) relapse. Increasing p16 expression correlated with an increased relative hazard of relapse, which suggested a continuous relationship of the data. Overall, tumor recurrence was observed in 34 (39%) of 88 cases. Thirteen (26%) of 50 cases with undetectable-to-low p16 expression (Group A) developed tumor recurrence. However, tumor recurrence was observed in 21 (55%) of 38 cases with p16 nuclear overexpression (Group B; P = 0.002; Fig. 3). Nevertheless, in a multivariate analysis adjusted for tumor grade, pretreatment PSA, and pathological stage, overexpression of p16 did not contribute prognostic information over pretreatment PSA, the strongest independent predictor of tumor recurrence.
Discussion

Normal prostate tissues display undetectable levels of p16 protein and INK4A exon 1α transcripts. It has been reported that p16 expression is low to undetectable in most normal human tissues analyzed (15, 27, 28). In support of these observations, there are relatively low and near-constant levels of p16 protein and mRNA throughout the cell cycle of normal lymphocytes in culture (29). The lack of p16 expression in hyperplastic glands parallels that of normal prostatic tissue. On the basis of these data, it is our hypothesis that these negative p16 phenotypes reflect basal physiological levels of p16.

Primary prostatic adenocarcinomas revealed two distinct p16 phenotypes. Most tumors (57% of cases) were found to have undetectable or very low levels of p16 protein expression (Group A). This was associated with low levels or absence of INK4A exon 1α transcripts. Another group of tumors (43%) showed elevated p16 protein expression (Group B) that was consistently associated with increased INK4A exon 1α transcripts. These findings suggest an up-regulation of the INK4A-α gene, resulting in p16 protein overexpression. Patients in Group B had a more aggressive course, demonstrated by high levels of pretreatment PSA ($P = 0.018$) and a sooner time to biochemical (PSA) relapse ($P = 0.002$). A worse prognosis for Group B is also revealed by the trending association of p16 overexpression with higher pathological stage.

The negative phenotype observed in Group A may correspond to the normal physiological state, reflecting low-to-undetectable p16 levels. Alternatively, it could be related to mutations affecting the INK4A gene, especially homozygous deletions, or methylation of the INK4A exon 1α promoter region. Nevertheless, it has been reported that these events are infrequent in prostate cancer (17–22). Furthermore, it seems that tumors with INK4A mutations have a more aggressive clinical course (30–32). Contrary to this, in the present study, we observed that Group A patients had a less aggressive behavior than Group B patients. For these reasons, we hypothesize that the negative phenotype observed in Group A is more likely a reflection of the normal physiological state.

**Fig. 2** Immunohistochemistry and in situ hybridization of human primary prostatic carcinomas. Consecutive sections of primary human prostate cancer tissues were processed as follows: A, immunohistochemical staining with an anti-p16 monoclonal antibody shows lack of immunoreaction in tumor cells and stromal elements; B, in situ hybridization reveals undetectable INK4A exon 1α transcripts in both tumor and normal stromal elements (antisense probe); C, immunohistochemical staining with an anti-p16 monoclonal antibody shows strong immunoreaction in the nuclei of tumor cells, as well as moderate cytoplasmic staining; and D, in situ hybridization shows high levels of INK4A exon 1α transcripts in tumor cells when the antisense probe is used. Normal glands (arrow) serve as an internal negative control in both immunohistochemical (C) and in situ hybridization (D) assays.
The up-regulation of the INK4A-α gene, resulting in the overexpression of p16 protein, may develop through different mechanisms. An association between increased p16 transcript and protein levels occurs in tumor cell lines and certain primary neoplasms that lack functional pRb (1, 33–36). Moreover, p16-mediated inhibition of cell cycle progression seems to be dependent on functional pRb (37, 38). These data support an association between p16 and pRb, in which the absence of functional pRb limits p16 activity and possibly promotes INK4A-α up-regulation. Alternatively, enhanced activation of the INK4A-α gene may occur. E2F1, a direct activator of the INK4A exon 1B promoter, does not seem to directly activate INK4A-α transcription (39). However, evidence does exist for an indirect effect; E2F1 overexpression has been reported to markedly increase p16 transcripts and p16-related cyclin-dependent kinase inhibitor activity (40). Overexpression of cyclin D1 and/or of Cdk4 may also influence p16 expression through a compensatory feedback loop in which deregulation of cyclin D/Cdk4 complexes results in increased levels of p16 protein (27, 41). In summary, it seems that an altered RB axis could trigger p16 overexpression in certain systems.

The association of p16 immunoreactivity with tumor grade, hormonal status, tumor stage, and preoperative PSA levels is shown in Table 1.

**Table 1** Association of p16 immunoreactivity with tumor grade, hormonal status, tumor stage, and preoperative PSA levels

<table>
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<tr>
<th>Number of Subjects</th>
<th>p16 immunoreactivity, n (%)</th>
<th>≤5%</th>
<th>&gt;5%</th>
<th>P</th>
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<tr>
<td>All subjects</td>
<td></td>
<td>50 (57)</td>
<td>38 (43)</td>
<td></td>
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<tr>
<td>Gleason grade&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>&lt;7</td>
<td></td>
<td>30 (37)</td>
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<td>6 (20)</td>
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<td>≥7</td>
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<td>18 (22)</td>
<td>11 (61)</td>
<td>7 (39)</td>
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<td>Hormonal status</td>
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<tr>
<td>Hormone-naive</td>
<td></td>
<td>54 (61)</td>
<td>40 (74)</td>
<td>14 (26)</td>
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<tr>
<td>Hormone-treated</td>
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<td>34 (39)</td>
<td>10 (29)</td>
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<td>53 (60)</td>
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<td>19 (36)</td>
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<td>35 (40)</td>
<td>16 (46)</td>
<td>19 (54)</td>
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<td>Pretreatment PSA (ng/ml)</td>
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<td>18 (20)</td>
<td>14 (78)</td>
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<td>19 (66)</td>
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<td>41 (47)</td>
<td>17 (41)</td>
<td>24 (59)</td>
</tr>
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</table>

<sup>a</sup> Grading is based on the radical prostatectomy specimen. Patients who had received neoadjuvant androgen therapy were unable to be graded consistently. Six patients did not have Gleason grade information and were excluded from this analysis.

**Fig. 3** Kaplan-Meier curves using the log-rank test for post-prostatectomy recurrence-free survival of patients with primary prostate cancer (n = 88) stratified by p16 expression (Group A or Group B). Patients with PSA relapse were classified as failures, whereas patients without evidence of disease or dead from other or unknown causes were censored. Median time to relapse was 46.25 months for Group B and has not been reached for Group A.
44), hyperthermia (45), and UV irradiation (46) has been reported to trigger p16 overexpression. In the present study, another type of cellular stress—androgen ablation—may account in part for this observed phenomenon. A subset of patients were treated with neoadjuvant androgen ablation, a strategy reported to decrease the incidence of positive surgical margins after prostatectomy (47). In the present study, p16 overexpression was observed in 71% of hormone-treated versus 26% of hormone-naive patients (P = 0.001). These data suggest that p16 expression may be enhanced by androgen depletion. Androgens are known to modulate the expression of other cyclin-dependent kinase inhibitors such as p27 and p21 (48). In addition, it has been reported that the presence of androgens triggers down-regulation of p16 in LNCaP cells (49), a finding consistent with our observation of p16 overexpression in cases of androgen ablation. It is also possible that the association between p16 expression and androgen ablation may, in part, reflect staging bias by clinicians. In this setting, patients thought to have advanced disease may have been treated with neoadjuvant therapy.

On the basis of the above referred data, it is our working hypothesis that p16 overexpression in prostate cancer represents an altered phenotype that identifies a subgroup of patients with a higher likelihood of postsurgical failure and tumor recurrence. In support of this postulate, a preliminary report in prostate cancer has demonstrated an association between p16 overexpression and poor outcome as related to biochemical failure (50). In addition, p16 overexpression has been associated with tumor progression and a poor prognosis in ovarian (51) and breast cancers (36). Although p16 acts as a negative cell cycle regulator, specific mechanisms may contribute to its altered expression, overcoming p16-mediated tumor suppressor activities. Ongoing studies may elucidate mechanisms of p16 overexpression relative to androgen depletion and/or alterations in the RB axis.

References


47. Witjes, W., Schulman, C., and Debruyne, F. Preliminary results of a prospective randomized study comparing radical prostatectomy versus radical prostatectomy associated with neoadjuvant hormonal combination therapy in T2–3 N0 M0 prostatic carcinoma. The European Study Group on Neoadjuvant Treatment of Prostate Cancer. Urology, 49 (Suppl.): 65s–69s, 1997.


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