Expression of Cell Cycle-regulated Proteins in Prostate Cancer

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ABSTRACT

Markers of cellular proliferation have proven to be useful prognostic markers in several tumor types. Recently, immunoreactivity for cyclins was found to provide an independent marker of tumor proliferation in breast cancer. In this study, we sought to determine the pattern of immunoreactivity for cyclins A, B, E, and Ki-67 in surgically resected prostate cancer and to determine their possible prognostic significance. Twenty-eight tumors of American Urological Association stages B and C were selected for study. Immunoreactivity for cyclins A and B was detected in most tumors and was present at significantly reduced levels as compared with breast cancer. Staining for cyclin E was present in four tumors and was present only in focal areas in two of the four. Such focal variation in expression of cell cycle regulators may reflect genetic instability in a tumor. Immunoreactivity for cyclins A and B was correlated with both Ki-67 index (the percentage of cells with Ki-67 immunoreactivity) and with each other. A Ki-67 index greater than 4.0 was associated with shorter time to prostate-specific antigen-detected relapse ($P = 0.026$). The fraction of cells staining for cyclins A and B divided by the fraction of cells staining for Ki-67 ($[A + B]/K$) was highly predictive of relapse, with values less than 0.50 associated with more rapid progression ($P < 0.001$). This latter result remained statistically significant after controlling for Gleason score by stratification. Our results suggest that immunoreactivity for markers of cellular proliferation may provide useful prognostic information in localized prostate cancer, and they need to be validated in a larger numbers of patients.

INTRODUCTION

Prostate cancer is now the most commonly diagnosed non-cutaneous malignancy in the United States, and its incidence is increasing (1). Relative to other common solid tumors, prostate cancer is often an indolent disease. Although treatment of early-stage prostate cancer is effective, it is associated with significant morbidity. Therefore, determining the optimal management of early-stage disease has become an increasingly significant clinical problem (2, 3).

The management of early-stage prostate cancer would benefit greatly from the establishment of reliable prognostic indicators. This would help to establish which patients stand to benefit most from aggressive initial therapy and which patients can be managed expectantly. Despite significant efforts over the last several years, very few disease markers have added significantly to the prognostic information provided by stage and grade.

Estimation of the proliferative behavior of a tumor is a well-established prognostic indicator in breast cancer. The use of one such measure, SFP, estimated by fluorescence-activated cell sorting analysis has been proposed as one factor to be considered in selecting patients for adjuvant therapy (4). SFP analysis can be performed on both frozen and paraffin-embedded, formalin-fixed tissues. Frozen tissue is not routinely available for study of prostate cancers, and SFP analysis of formalin-fixed tissue may be compromised by nuclear fragmentation or inclusion of nontumor tissue.

Because immunostaining can be performed on formalin-fixed tissue, these methods are better suited to the analysis of routine prostate specimens. Additionally, unlike SFP, immunostaining allows the simultaneous assessment of morphology so that the data are not confounded by the presence of significant amounts of stromal tissue. Immunostaining for various cellular proteins whose level is increased in dividing cells has also been used as a method to measure the proliferative capacity of a tumor. Examples of such proteins include PCNA (5) and Ki-67 (6–9). Analysis of these proteins has been shown to correlate well with the proliferative index as determined by SFP. Because both PCNA and Ki-67 are elevated throughout the cell cycle, they provide no information regarding the stage of the cell cycle or the kinetics of the population of cycling cells.

Cyclins are proteins that vary in their relative abundance during different stages of the cell cycle. Different cyclins can activate various kinases (cyclin-dependent kinases), and specific cyclin/cyclin-dependent kinase complexes are required for passage out of a particular stage of the cell cycle. Cyclin E is present in G1 and early S, cyclin A in S and G2, and cyclin B in late G2. Thus, each of these three cyclins can be used as markers for various stages of the cell cycle. In a study in breast cancer, we demonstrated that cyclin levels detected by immunostaining correlated well with staining for Ki-67 and were also well correlated with each other (10). In addition to providing increased reliability to the measurement of the proliferative index, cyclin staining provided additional information about cell cycle kinetics. We, therefore, sought to extend our observations to prostate cancer.

PATIENTS AND METHODS

Selection of Patients. Twenty-eight patients who underwent radical retropubic prostatectomy were retrospectively selected for analysis. Preoperative PSA levels were available only for those patients undergoing surgery in 1989 or later. Pathology was reviewed by a single genito-urinary pathologist (C. C.). Pathological stage, Gleason score, preoperative PSA, and time to relapse for each of the tumors studied are summarized in Table 1. Patients with no signs of relapse were followed for an average of 62 months (range, 32–93 months), whereas patients whose disease relapsed did so in an average of 28 months (range, 3–72 months). No patients received postoperative radiation therapy, chemotherapy, or hormonal therapy.

Definition of Relapse. Relapse was defined by either radiographic and/or pathological evidence of progressive disease, development of bone metastases as determined by radionuclide bone scanning, or biochemical evidence of disease progression. Biochemical relapse was defined as a PSA that had more than doubled over baseline on two consecutive measurements and was at least 0.4 ng/ml. Twelve patients had relapsed, as determined by rising PSA only, whereas three patients (patients 10, 19, and 28) had both rising PSA and radiographic or pathological evidence of disease progression.

Immunocytochemical Staining for Cyclins. The antibodies used were polyclonal rabbit antisera against bacterially expressed recombinant cyclin A.
CYCLIN EXPRESSION IN PROSTATE CANCER

Table 1  The staining indices for indicated proliferation-related proteins is presented along with the pathological grade (Gleason score), the preoperative level of PSA, the clinical stage, and time to relapse following radical prostatectomy

<table>
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<th>Case no.</th>
<th>Gleason score</th>
<th>Pre-op&lt;sup&gt;a&lt;/sup&gt; PSA (ng/ml)</th>
<th>AUA Stage</th>
<th>Time to relapse (mos)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Ki-67</th>
<th>Cyclin A</th>
<th>Cyclin B</th>
<th>Cyclin E</th>
<th>(A + B)/K</th>
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<sup>a</sup> Pre-op, preoperative; AUA, American Urological Association; NA, not available.

<sup>b</sup> The number of months followed by a + sign indicates that the tumor has not relapsed in the indicated number of months of follow-up.

(from Dr. Jonathan Pines, CRC Laboratory, Cambridge, United Kingdom) and mouse monoclonal antibodies GNS1 to cyclin B and HE12 to cyclin E (from Dr. Ed Harlow, Massachusetts General Hospital, Boston, MA). Monoclonal antibodies for Ki-67 (MB 1) were purchased from AMAC, Inc. (Westbrook, ME). The use of these antibodies in paraffin sections and their validation as antibodies that detect the appropriate cell cycle-regulated proteins has been described (10).

Staining indices are defined as the percentage of tumor cells displaying immunoreactivity. They were determined by examining areas with homogeneous distribution of tumor cells, the staining of which was representative of the tumor section (surveyed at x 100). Cell counts were performed at x 400 using a 10 x 10 grid in the eye-piece. The total number of tumor nuclei in the area of the grid (the denominator) was estimated by counting the nuclei in one-fifth of the squares distributed throughout the grid and multiplying by 5. All positively staining cells in the area of the grid were counted for the numerator. Adjacent fields were counted until at least 1000 tumor cells were surveyed.

Statistical Analysis. Time to failure (relapse) was measured from the date of radical prostatectomy to the date of failure or the date of last follow-up without evidence of failure. Subgroups were defined by splitting each covariate approximately on the median. Kaplan-Meier curves were calculated for each group and compared via the log rank test. Stratified log rank tests were used to evaluate the effects within subgroups defined by Gleason score (5–6 versus 7–10).

RESULTS

Twenty-eight tumors derived from radical prostatectomy specimens were analyzed for immunoreactivity to Ki-67, cyclin A, cyclin B, and cyclin E. The results are summarized in Table 1. Cyclin A, cyclin E, and Ki-67 were detected in the nuclei (Fig. 1), whereas cyclin B was detected in the cytoplasm (data not shown). The intensity of staining shown in Fig. 1 was representative of that seen with all tumors examined. The HE12 antibody against cyclin E cross-reacts with an endothelial protein that is exclusively localized in the cytoplasm, which results in the tumor vessels displaying high immunoreactivity, as with Factor VIII. However, as shown earlier, this cytoplasmic immunostaining was easily distinguished from the nuclear immunoreactivity of authentic cyclin E.

Immunoreactivity for Ki-67 and cyclins A and B were well correlated with each other (Table 2). The correlation between the cyclin A index and the Ki-67 index was 0.73, even after eliminating an outlier (case 28) that had an extremely high staining index for all markers. This is consistent with our previous observation in breast cancer that the cyclin A staining provides a second proliferation marker. A perfect correlation of 1.0 between cyclin A and Ki-67 is not expected because they are expressed during different periods in the cell cycle. A moderate positive correlation was seen between the cyclin B index and the cyclin A and Ki-67 indices.

Cyclin E was either absent or present at very low levels, with the exception of two cases: case 28, a rapidly fatal tumor; and case 9. In these two tumors, cyclin E expression was focal in some areas and non-focal in others. In case 28, there were focal areas with almost 100% of the tumor cells expressing cyclin E, a staining index that far exceeded that of Ki-67 or cyclin A (Fig. 1). In this particular tumor, the staining indices in these foci are not comparable to the more general staining pattern seen in the rest of the tumor (compare Fig. 1 with the staining indices reported for tumor 28 in Table 1). Such foci of aberrant expression of cell cycle regulators could indicate genetic instability in the tumor, which may itself account for the rapid progression of disease in this case.

The histopathological grade of a prostate cancer (often expressed as a Gleason score) is a well-established prognostic indicator. Cancer volume and cancer doubling times have also been proposed as prognostic indicators (11). For this study, we selected patients with tumors that were in the mid-range of Gleason scores, with about one-half the tumors having Gleason sums of 6 or 7. No tumor had a Gleason score less than 5. The Ki-67 index was positively correlated with the Gleason score (r = 0.55; if case 28 is eliminated, r = 0.57).

Kaplan-Meier plots of probability of disease-free survival versus Gleason score, Ki-67 index, and (A + B)/K index are shown in Fig. 2. The Gleason score was associated with time to relapse (P = 0.066).
Fig. 1. Aberrant focal overexpression of cyclin E relative to cyclin A or Ki-67 in one prostate cancer. Adjacent sections from the same block of tumor from case 28 were stained with anti-Ki-67 (A), anti-cyclin A (B), and anti-cyclin E (C) antibodies. Positive stain is indicated by the dark brown color in the nucleus. Counterstain was with methyl green (A and B) and hematoxylin (C).
The probability of disease-free survival at 50 weeks was 0.7 with a Gleason score of 5 and 6 and 0.4 with a score of 7 and greater. Ki-67 index was also associated with time to relapse ($P = 0.026$). The probability of disease-free survival at 50 weeks was 0.7, with a Ki-67 index of less than 4, and 0.35 with a Ki-67 index of 4 and greater than 4. Pathological stage and immunoreactivity for cyclins A and B were not significantly associated with time to relapse. In a stratified analysis controlling for Gleason score, Ki-67 was not found to be an independent predictor of time to progression ($P = 0.12$).

Since cell cycle stage-specific markers yield additional information about the position of the tumor cells in the cell cycle, we analyzed the predictive ability of several composite indices based on these markers. The most powerful predictor of time to relapse was a ratio of cells with immunoreactivity to cyclins A or B to those with immunoreactivity for Ki-67 [(A + B)/K; $P = 0.00007$]. The probability of disease-free survival at 50 weeks was 0.85 for tumors with (A + B)/K of 0.5 and greater and 0.2 for tumors with (A + B)/K of less than 0.5. The association between (A + B)/K and outcome was not continuous. Rather, there appeared to be a marked difference in outcome for tumors with a ratio below 0.5 and tumors with a ratio above 0.5. Other indices, such as cyclin A index/Ki-67 index and cyclin B index/Ki-67 index, were not as powerful a predictor of prognosis. (A + B)/K retained its predictive power, even if case 28 was not considered.

**DISCUSSION**

In a previous study, we detailed a method for analyzing immunoreactivity to cell cycle proteins in paraffin-embedded, formalin-fixed tissues (10). Immunoreactivity for the various cyclins correlated well with each other and with the cell proliferative index as determined by SPF. We have now extended our analysis to localized prostate cancer. Stage D tumors will be studied separately in the future.

Cyclins A, B, and E, like other markers of cellular proliferation, are far less abundant in prostatic carcinoma than in breast carcinoma. As shown in Table 3, the mean staining index for each of the markers was lower in the prostate cancer survey than in the breast cancer survey. Although the exact magnitude of the difference may be affected by selection bias, the nature of the difference is consistent with the reported mitotic indices for these two types of cancer.

In prostate tumors, immunoreactivity for cyclin A appears to correlate well with immunoreactivity for Ki-67 in the twenty-eight tumors we examined. As was noticed in breast cancers, staining for cyclin B correlates less well with the other proliferation markers. A technical factor that might account for the poorer correlation is the small window of time in the cell cycle when cyclin B is expressed. Thus, a small fraction of tumor cells stain for the protein at a given time, resulting in larger sampling errors when surveying only 1000 cells. Incidentally, the same problem is to be expected when correlating the mitotic indices of slowly proliferating tumors with staining for Ki-67 or cyclin A.

In contrast to breast cancers, cyclin E was rarely expressed in prostate cancers. However, a few tumors exhibited intense local immunoreactivity for cyclin E in some areas. Keyomarsi et al. (12) have reported high levels of cyclin E in seven of seven prostate tumors by immunoblotting of tumor protein extracts. They reported that the overexpression of cyclin E in tumor tissue was greater than expected.
from the level of expression of the proliferation marker PCNA, consistent with our observation that focal overexpression of cyclin E was not accompanied by overexpression of Ki-67, cyclin A, or cyclin B. The precise cause of this focal overexpression and its clinical implications deserve further study. Since cyclin D1 overexpression may contribute to the pathogenesis of breast cancer (13), it is conceivable that overexpression of cyclin E may also be an important event in cancer progression. Increased levels of cyclin E shorten the G1 phase in cultured cells (14, 15) and promotes DNA replication in in vitro reactions using Xenopus egg extracts (16). Therefore, subclones of tumor cells with elevated cyclin E expression may eventually acquire a growth advantage and contribute to tumor progression.

As with most outcome studies in prostate cancer, we have chosen to use biochemical relapse as a clinical end point because of the long natural history of the disease. Although biochemical relapse can be expected to correlate with more clinically relevant end points such as survival, this has not yet been demonstrated conclusively.

Previous studies using PCNA immunoreactivity to study tumor proliferation have been criticized because of the variability in antigen preservation with routine fixation and immunostaining techniques. Other studies have examined Ki-67 immunoreactivity in prostate carcinoma (6—9). In all of these studies, as well as in the present study, there was a correlation between increased Ki-67 immunoreactivity and increased Gleason score. However, attempts to correlate Ki-67 index in prostate tumors with clinical outcome have met with mixed results (6—9). Taken together with the previous data, our study also suggests that there is not a marked correlation between Ki-67 immunoreactivity and clinical outcome that is independent of the Gleason score.

In contrast, the ratio of the fraction of cells with immunoreactivity to cyclins A or B to the fraction of cells with immunoreactivity to Ki-67 [(A + B)/Ki-67] may provide significant additional prognostic information. Although there are only small numbers of patients in the current study, a ratio less than 0.5 proved to be a powerful predictor of time to biochemical relapse. These results need to be confirmed in a larger population of patients. If confirmed, this information may be useful in selecting patients who are at high risk for relapse following radical prostatectomy and are, therefore, candidates for early intervention with other therapeutic modalities. Our results also suggest that performing a similar analysis on needle biopsy specimens should also be considered.

Since there is a trend toward a worse prognosis with a high Ki-67 index, it is expected that a ratio with the Ki-67 index in the denominator will be inversely correlated with prognosis. However the 
\[(A + B)/K\] index is a more powerful predictor of probability of relapse than only \(1/K\) or \(A/K\) or \(B/K\). If these results are confirmed in a larger trial, one might conclude that tumors with a large proportion of proliferating cells expressing cyclins A or B (i.e., in S-phase or G2-M phase) are less aggressive than tumors with most of their proliferating cells in G1. Perhaps tumor cells in G1 are more likely to express genes required for invasion and metastasis than those in other phases of the cell cycle. Alternatively, prostate cancer cells could have error-prone DNA replication, lost replication error-induced check-point function, or be more likely to undergo apoptosis rather than DNA repair following replication errors. We speculate that tumor cells expressing cyclin A or B (therefore, in S or G1) would then be more likely to lose viability, explaining why a high \((A + B)/K\) index predicts a better prognosis.

A detailed study of cell cycle kinetics that measured both Ki-67 expression and apoptosis demonstrates that progression of prostate cancer is marked initially by an increase in tumor cell proliferation accompanied by a compensatory increase in cell death (17). In the later stages of the disease, the rate of apoptosis decreases relative to the rate of proliferation, resulting in a shorter tumor doubling time. Thus, an effective prognostic indicator in prostate cancer should have to take cell death rates into account. In this context, the prognostic success of the \((A + B)/K\) index supports our speculation that this index somehow measures not only tumor cell proliferation but also tumor cell apoptosis. Future experiments will address this question directly.

REFERENCES


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