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ABSTRACT

Purpose: The quest for prognostic molecular markers in prostatic carcinoma is still in progress. Many proteins have already been screened by immunohistochemistry with the aim to find the most reliable indicator of progressive disease. In this study, we evaluated the expression of pRb2/p130, p107, p27kip1, p53, mdm-2, and Ki-67 (MIB-1) by immunohistochemistry in 24 prostate carcinomas compared with the paired expression of normal prostates.

Experimental Design: Expression of the different proteins in normal and pathological specimens was evaluated by the Wilcoxon test. A matrix of correlation (Spearman coefficient) was used to evaluate the possible association in expression among the different proteins. Logistic regression analysis was used to test the multivariable prognostic value of the levels of protein expression for the probability of disease development.

Results: p53 and Ki-67 (MIB-1) showed a higher expression in cancer than in normal tissue (P = 0.006 and <0.001, respectively), pRb2/p130, p107, and p27kip1 showed an overall lower expression in cancer, but the difference between cytoplasmic and nuclear expression was always higher for cancer (PS, from <0.001 to 0.016). mdm-2 expression was lower in cancer, but the difference between cytoplasmic and nuclear expression was not significant (P = 0.571) when compared with that in normal tissue. A positive correlation between p27 and pRb2/p130 levels expressed, in normal and cancer counterparts in the same sample, as the difference between cytoplasmic and nuclear protein concentrations (P = 0.045) was found. Additionally, p107 expression showed an inverse correlation with Ki-67 (MIB-1) expression in the most aggressive tumors (P = 0.046). Logistic regression output showed that Ki-67 (MIB-1) and pRb2/p130 (expressed as differences between cytoplasmic and nuclear concentrations) were the variables associated with a higher risk of cancer. The highest value was reported for Ki-67 (MIB-1) (odds ratio, 2.11), followed by pRb2/p130 (odds ratio, 1.01). pRb2/p130 alone was associated with a sensitivity (rate of cases having a posterior probability of disease ≥0.5) of 61% with a false positive rate of 22%. Ki-67 (MIB-1) alone yielded a sensitivity of 69% and a false positive rate of 14%. The combined model (Ki-67 + pRb2/p130) yielded a sensitivity of 83% with a false positive rate of 17%.

Interestingly, one specimen in which we also found a high-grade prostatic intraepithelial neoplasia showed the progressive loss of pRb2/p130 from normal prostatic cells to prostatic intraepithelial neoplasia cells, suggesting that in prostatic cancer, lack of expression of the tumor suppressor gene pRb2/p130 could be involved in the progression of the disease, from an early stage.

Conclusions: This study showed that all of the proteins but mdm-2 were expressed at a different rate in normal and pathological prostate specimens. Multivariate analysis showed that pRb2/p130 and p107 may be involved in the pathogenesis and progression of prostate cancers, and that the expression of the retinoblastoma-related protein pRb2/p130 along with Ki-67 (MIB-1), expressed as differences between cytoplasmic and nuclear concentrations, could be considered new parameters to be evaluated in discriminating patients at a higher risk for prostate cancer.

INTRODUCTION

Prostate cancer is the most commonly diagnosed malignancy and the second leading cause of cancer mortality in...
American males (1). Cap is a rare disease before age 40; however, its incidence increases by the age of 70 (2). The biology of early prostate cancer is variable and extends over many years, with some tumors progressing slowly or not at all, whereas others may progress more rapidly and be fatal after a few years. Prostate cancer is not only significant for its lethality but also for the extremely high morbidity associated with it. Screening programs have been implemented to diagnose men with Cap in its early stages and, although only a subset of men diagnosed with localized Cap will end up with metastasis, more precise markers are still needed so that appropriate treatment decisions can be made for individual Cap patients. More specific biological criteria should be addressed to better clinically differentiate patients with more aggressive versus indolent prostate carcinoma.

The pelvic lymph nodes and bones are the major sites of prostate cancer metastases. Pain is the dominant symptom that patients with metastatic prostate cancer experience, and progression of disease is in the form of increasing prostate-specific antigen levels and/or increase in size or number of bony or soft tissue lesions is eventually seen in most patients with prostate cancer treated with androgen ablation (2).

One marker that has been very well studied in different tumors is the p53 tumor-suppressor gene (TP53; Ref. 3). p53 has been found mutated and associated with poor prognosis in prostatic carcinoma (4–8). However, there is still a discrepancy in the frequency of p53 mutations in Cap (6–50%) and on its prognostic role (9–13).

The p53 protein is activated in response to physiological stress resulting either in G1 arrest of cells or in apoptosis. mdm-2 plays a pivotal role in this regulatory process. These two proteins form an autoregulatory feedback loop, in which p53 positively regulates mdm-2 levels and mdm-2 negatively regulates p53 levels and activity. The induction of mdm-2 results in the inhibition of p53 transcriptional activity and the degradation of the p53 protein (14). Alteration in the p53 and mdm-2 genes have been rarely found in clinically localized, stage B prostate carcinomas. In contrast, stage C and D prostate carcinomas showed accumulation of p53 protein in 33–66% of cases (15). Therefore, because alterations in p53 function are infrequent in clinically localized prostate cancers but are more common in advanced cancers, p53 cannot be considered an early marker for prostatic cancer.

Cell cycle progression is regulated by a series of Cdks. Cdks are regulated both by activating and inhibitory phosphorylations as well as by Cdk inhibitors, which bind the cyclin-Cdk complexes and inhibit their activity (16). There are two groups of Cdk kinase inhibitors, the Ink family (inhibitors of Cdk4) and the Cip/Kip family (inhibitors of cdk2). The Cip/Kip family includes p21, p27\(^{kip1}\), and p57, which are structurally related. p27\(^{kip1}\) is present in abundance in quiescent cells, and its expression diminishes, primarily by proteasome degradation, immediately before S-phase entry. Loss of p27\(^{kip1}\) expression has been shown to be a negative prognostic marker in mammary, gastric, pulmonary, esophageal, and colorectal carcinomas (17–23). Loss of p27\(^{kip1}\) expression has been recently shown to be a negative prognostic marker in prostatic carcinoma (24–28).

Proliferative tumor activity has been measured by determining the S-phase fraction of DNA by flow cytometry, but the efficacy and significance of such measurement is still unclear (29). One of the most reliable markers to estimate proliferation is Ki-67 (MB-1). Its positivity has been associated with Gleason score, extension outside of the prostate, and seminal vesicle involvement in a series of 208 prostate carcinomas (30). A high Ki-67 (MB-1) proliferative rate and a high immunoreactivity for bcl-2 have been associated with increased prostate carcinoma recurrence.

Despite recent progress in molecular medicine, there is still a paucity of data regarding the involvement of other cell cycle-regulatory proteins such as those of the RB family in the pathogenesis of prostatic cancer. The RB gene family is composed of three members, the RB gene, which is one of the most studied tumor suppressor genes, and two other related genes, RB2/p130 and p107. The proteins encoded by these genes are structurally and functionally similar to each other and play a pivotal role as negative regulators of cell proliferation (31). However, the three proteins exhibit unique growth-suppressive properties in specific cell lines, which suggests that, although the different members of the RB family may complement each other, they are not fully functionally redundant (32, 33). RB2/p130 maps to human chromosome 16q12.2, an area that is frequently altered in several human neoplasias including breast, ovarian, hepatic, prostatic, and endometrial carcinomas (34). Others, as well as ourselves, have recently reported that RB2/p130 is involved in the pathogenesis and progression of lung cancer (35–38), nasopharyngeal carcinoma (32, 39), and lymphomas (40) and is a strong predictor of clinical outcome in endometrial carcinomas (41) as well as in choroidal melanomas (42).

The present study was undertaken to investigate whether the degree of pRb2/p130 expression alone, or in combination with that of p107, p27\(^{kip1}\), p53, mdm-2, or Ki-67 (MB-1), is a predictor of the clinical behavior in prostatic tumors. We studied a panel of 24 patients with prostatic carcinoma who did not receive radiation, hormonal therapy, or chemotherapy before surgery. We have evaluated the relationship between expression of these proteins and clinicopathological parameters such as age, prostate volume, tumor volume, surgical prostate margins assessed by ink staining, prostate weight and size, pathological stage, Gleason score, capsular extension, and seminal vesicle involvement.

**MATERIALS AND METHODS**

**Patient Populations and Clinicopathological Data.** Sections from 24 consecutive paraffin-embedded prostate gland adenocarcinomas were obtained from patients who underwent retropubic surgical resection as a first treatment at the Department of Pathology, MCP Hahnemann University, Philadelphia, PA.

Prostate glands were inked and submitted for routine histopathological examination. Staging was carried out according to the TNM classification, and tumors were graded according to the Gleason system (43). Each prostate gland was assessed for extension of the tumor to an inked specimen margin as well as...
extracapsular extension into periprostatic tissue. Only patients with primary CAP who had not undergone any previous irradiation or chemotherapeutic treatment were included in the study. Two experienced pathologists (F. U. G. and G. G. G.) blindly and independently confirmed the histological diagnosis of each prostatic lesion and agreed on the Gleason grading (Table 1). The median age of the patients was 59 years (range, 44–77 years). Our patients showed advanced local growth of tumor. In fact, the Gleason score was between 6 and 9, with a higher prevalence of 7, 8, and 9. Eleven (46%) of 24 tumors were not organ-confined (stage T2), and the median prostate weight was 38.5 g (range, 14–79 g) with a median prostate volume of 40 mm$^3$ (range, 3.8–262.1 mm$^3$), and a tumor volume of 9.17 mm$^3$ (range, 1.7–30.2 mm$^3$). All of the patients showed a perineural invasion and 13 (56%) of them did not show positivity of the surgical margins assessed by ink staining. Additionally, the seminal vesicles were involved in four (17%) of the 24 cases.

**Immunohistochemistry.** A total of 24 formalin-fixed, paraffin-embedded tumor tissue samples and adjacent uninvolved prostatic gland were processed. Sections of each specimen were cut at 3 μm, mounted on glass, and dried overnight at 37°C. All of the sections were dewaxed, rehydrated, quenched in 0.5% hydrogen peroxide, and microwave-pretreated in 10 mM citrate buffer (pH 6.0) for 15 min at 650 W for p107, 15 min at 650 W for pRB2/p130, 10 min at 650 W for p27$^{kip1}$, 10 min at 650 W for mdm-2, and 20 min at 650 W for p53 and Ki-67 (MIB-1). After blocking with normal serum for 1 h at room temperature, our rabbit polyclonal antibody against p107 was incubated with tissue sections at a 1:200 dilution at 4°C overnight. The mouse monoclonal antibody against p27$^{kip1}$ (clone DCS-72.P6; Neomarkers, Inc., Union City, CA) was incubated with tissue sections at a 1:150 dilution at 4°C overnight. The mouse monoclonal antibody against p53 (clone DO7; Dako, Carpinteria, CA) was incubated with tissue sections at a 1:3200 dilution for 3 h at room temperature. The mouse monoclonal antibody against Ki-67 (MIB-1; Beckman Coulter, Inc., Fullerton, CA) was incubated with tissue sections at a 1:80 dilution for 3 h at room temperature. Negative control slides were also processed simultaneously using a nonspecific immunoglobulin IgG (Sigma Chemical Co., St. Louis, MO) at the same concentration as the primary antibody. The positive immunostaining of infiltrating lymphocytes represented an internal positive control for preservation of antigenicity in the sections examined. All of the slides were processed using the ABC method (Vector Laboratories, Burlingame, CA). Diaminobenzidine was used as the final chromogen and Gill’s hematoxylin was used for counterstaining.

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The patients were divided into three treatment groups: (1) patients in whom surgical treatment was performed with no systemic or local recurrence or relapse; (2) patients in whom surgical treatment was performed and who subsequently developed one or more recurrences; and (3) patients who were never treated surgically. Contingency tables were analyzed by the Fisher’s exact test. A p value < 0.05 was considered significant. The Wald test was used to assess the significance of the logistic model, and the odds ratio (OR) and its 95% confidence interval (CI) were calculated. The area under the receiver operating characteristic (ROC) curve was used to evaluate the ability of the model to predict the disease. Sensitivity, specificity, and false-positive rate of each protein and of the combined model to predict the disease were also tested. Contingency tables were created using a posterior probability $\geq 0.5$ as the cutoff point.

**Table 1 Patient populations and clinicopathological data**

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RESULTS

All of the studied proteins showed a different expression pattern in pathological specimens when compared with normal tissue. Considering the difference between intracytoplasmic and nuclear proteins concentrations, pRb2/p130 and p107 proved to be differently distributed in normal and pathological specimens. The difference was considered the eligible variable for further analysis. p27, p53, and Ki-67 (MIB-1) expressions were higher in the pathological cases.

**p53, mdm-2, and p27kip1 Expression in Prostate Tumors.** The expression levels of p53, mdm-2, p27kip1 and Ki-67 (MIB-1) in prostate tumors were determined by immunohistochemistry.

In normal prostatic cells, the level and intensity of p53 staining was uniformly low in all of the samples examined. High expression levels of p53 were detected in only 2 (8%) of 24 samples that were considered to be pathological stage T3 and with a high Gleason score (Fig. 1, A and B). This result is in accordance with previous reports that describe p53 mutations as a late event in prostate carcinogenesis (10–12, 15, 44). In fact, very frequent p53 mutations have been reported in metastatic prostate carcinoma and in matched primary tumors (45).

In normal prostatic cells a high immunostaining of mdm-2 was detected in all of the samples (Fig. 1C). Conversely, a low immunoreactivity for mdm-2 was recorded for cases with higher Gleason pattern (4–5) and high pathological stage T3 (Fig. 1D). mdm-2 immunoreactivity was lower in cancer specimens when compared with normal cells even if the difference between mdm-2 normal nuclear and cytoplasmic and cancer nuclear and cytoplasmic expression did not reach any statistical significance in a Wilcoxon signed-ranks test ($P = 0.571$).

In normal prostatic cells, the level and intensity of p27kip1 immunostaining was uniformly high (Fig. 1E). p27kip1 immunoreactivity was concentrated within the nucleus, but diffuse cytoplasmic staining was also observed. In contrast, low levels of p27kip1 expression were found in prostate cancer (Fig. 1F) and correlated with more aggressive prostate tumors, as reported previously (46–48).

**RB-related p107, pRb2/p130, and Ki-67 (MIB-1) Expressions in Prostate Tumors.** Complete lack of nuclear expression of p107 in tumoral prostatic cells (IS, 0), was found in 13 cases (54%). Six cases (25%) showed low nuclear staining (IS, between 30 and 60), four cases (17%) had an IS between 80 and 120 and one case (4%) had a high score such as 180. All of the cases showed p107 cytoplasmic staining in carcinoma cells, varying between 40 and 360. Nine cases (38%) showed high cytoplasmic positivity for p107 in tumoral cells (IS, between 240 and 360), 12 cases (50%) showed a cytoplasmic IS between 80 and 180, and...
3 cases (12%) an IS score between 40 and 60. Fig. 2A shows p107 immunoreactivity in normal prostatic glands, whereas Fig. 2B shows the weak p107 nuclear immunostaining observed in the matching tumor. CAP showed a lower expression of both intracytoplasmic and nuclear p107 levels, and the difference between cytoplasmic and nuclear levels was much higher for cancer than for normal cells (\( P < 0.004 \)).

Low level of pRb2/p130 nuclear expression in carcinoma cells (IS between 20 and 40) was found in 6 (25%) of 24 samples examined. Complete absence of nuclear pRb2/p130 expression (IS, 0), was found in 6 cases (25%). Six other cases (25%) had a score between 60 and 120, five cases (21%) had a score between 180 and 240, and only one case (4%) a high score of 360. Normal prostate glands showed nuclear staining (Fig. 2C). The majority of the cases (95.6%) also showed a prevalent pRb2/p130 cytoplasmic staining in cancer cells (Fig. 2D). The difference between the expression of pRb2/p130 in the nucleus and the cytoplasm of the carcinoma cells was determined to be statistically significant using a Wilcoxon signed-ranks test (\( P = 0.009 \)). Additionally, the difference of cytoplasmic and nuclear pRb2/p130 expression between normal and cancer cells was highly statistically significant using the Wilcoxon signed-ranks test (\( P = 0.016 \)). Interestingly, one specimen, in which there was also a HG-PIN that was stained for pRb2/p130, showed a progressive loss of pRb2/p130 from normal prostatic cells to PIN cells (Fig. 2E).

Correlation matrix (Spearman coefficient) showed a positive relationship between p27 and pRb2/p130 levels in normal and cancer fields in the same specimen, expressed as the difference between cytoplasmic and nuclear protein concentration (\( P < 0.045 \)). This correlation was attributable only to the intracytoplasmic levels of the proteins (\( P < 0.030 \)). In other words, the intracytoplasmic levels of the proteins were correlated, but this correlation disappeared in the presence of cancer.

The Ki-67 (MIB-1) proliferation indicator was highly expressed in all tumoral cells (Fig. 2F) and, conversely, was low in the normal matched tissues, as reported previously (30). In cancer, low nuclear staining of p107 was associated with high Ki-67 (MIB-1) immunoreactivity (\( P < 0.046 \)). No correlation was found between p53 and mdm-2 expression and either p107 or pRb2/p130 expression patterns.

**Multivariate Analysis.** A stepwise logistic regression output that expresses the risk of cancer in relation to Ki-67 (MIB-1) immunoreactivity is reported in Table 2. Only the significant values and variables have been reported in the final analysis. As shown, Ki-67 (MIB-1) and pRb2/p130 (ex-
pressed as differences between cytoplasmic and nuclear concentrations) are the variables associated with a higher risk of cancer. The highest value was reported for Ki-67 (MIB-1), (OR, 2.11) followed by that of pRb2/p130 (OR, 1.01). pRb2/p130 alone was associated with a sensitivity (rate of pathological cases having a posterior probability ≥0.5) of 61% with a false-positive rate of 22%. Ki-67 (MIB-1) alone yielded a sensitivity of 69% and a false-positive rate of 14%. The combined model (Ki-67 + pRb2/p130) yielded a sensitivity of 83% with a false-positive rate of 17% (Table 3).

Correlation of pRb2/p130, p107, p27kip1, p53, mdm-2, and Ki-67 (MIB-1) Expression with Clinicopathological Parameters. The relationship between all of the proteins studied with a series of clinicopathological parameters (age, Gleason score, pathological stage, extracapsular extension, prostate weight and volume, tumor volume, perineural invasion, surgical prostate margins assessed by ink staining, and seminal vesicle involvement) was investigated in 24 prostate gland tumors. The median age of the patients was 59 years (range, 44–78 years). These patients showed advanced local growth of the tumor. In fact, the Gleason score was between 4 and 9, with a prevalence of 7 and 8. Eleven (46%) of 24 patients had extracapsular extension, and the median prostate weight was 38.5 g (range, 14–79 g) with a median prostate volume of 40 mm³ (range, 3.8–262.1 mm³), and a tumor volume of 9.17 mm³ (range, 1.7–30.2 mm³). All of the patients showed a perineural invasion and thirteen (54%) of them did not show positivity of the surgical margins assessed by ink staining. Additionally, the seminal vesicles were involved in 4 (17%) of the 24 cases.

Capsular extension, surgical prostate margins, and tumor volume did not correlate with the proteins’ expression and were deleted from the statistical analysis.

Considering the low number of cases, we could not statistically compare the proteins’ expression patterns with the Gleason score. However, we found an inverse trend between nuclear pRb2/p130 expression and tumor malignancy. In fact, all of the cases classified with a very high Gleason score showed high cytoplasmic immunoreactivity.

p107 also showed a negative trend between nuclear expression pattern and tumor malignancy. In fact, in malignant prostatic carcinoma cells, p107 was expressed mostly in the cytoplasm and in a relatively high number of specimens (61%) with a high score.

DISCUSSION

In the United States, prostate cancer is still the most commonly diagnosed cancer and the leading cause of death by cancer in men. The evolution of this type of cancer is variable and may extend over many years. Additionally, prostate cancer is significant not only for its lethality but also for its morbidity. Pelvic lymph nodes and bone are the major sites of prostate metastases. For many reasons, until recently, there has been no successful standard treatment for this disease, and most physicians treat progressive hormone-resistant metastatic prostate cancer with palliative radiotherapy and supportive care (2, 49). Many studies are still in progress to clarify the role of different cellular proteins in the genesis and progression of CAP. In the present study, we evaluated the expression of pRb2/p130, p107, p27kip1, p53, mdm-2, and Ki-67 (MIB-1) by immunohistochemistry in a panel of 24 prostate carcinomas compared with the paired expression in normal prostate. The analysis of p53, mdm-2, and p27 immunostaining confirmed previous reports that indicated p53 and mdm-2 positivity as markers of progressed disease (10–12, 15, 44, 50) and the lack of p27 staining as correlating with more aggressive prostate tumors (46–48). The analysis of the proliferation marker Ki-67 showed high immunoreactivity in tumoral cells when compared with the normal matched tissue, as reported previously (30).

The RB susceptibility gene encodes a nuclear phosphoprotein, which is involved in cell cycle control and cell differentiation. The RB protein is mutated or absent in a variety of human malignancies. Its role as a molecular marker for clinical tumor behavior is under present extensive investigation. In different studies, prostate carcinoma had an incidence of aberrant RB gene expression ranging from 9 to 60% (51–53).

Additionally, numerous studies have coupled immunohistochemical analysis to the use of molecular biology techniques such as genomic DNA mutational analysis. Mutations of tumor suppressor genes are critical genetic alterations occurring during the genesis and progression of human cancer, and consequently are candidates for use as surrogate end point biomarkers.

Loss of the RB1 gene is an important event in the initiation and progression of many tumors. In a recent study, prostate tissue from 43 patients with prostate cancers and 10 with BPH were screened for loss of heterozygosity of the RB1 gene. Loss of heterozygosity was found in 24 (60%) of 40 informative patients with cancer. Loss of RB1 occurred with a similar frequency in early-stage and low-grade cancers as in more advanced cancers. Loss of RB1 was also found in one patient with BPH. Expression of pRB was completely absent from seven cancers and markedly reduced in the other two, whereas nuclear pRB staining was always present in areas of BPH, whether alongside cancer-containing tissue or with BPH alone. These data demonstrated that the loss of RB1 could be an early event in prostatic tumorigenesis (51).

On the other hand, in a more recent study the immunohistochemical expression of RB and p53 proteins in HG-PIN, benign prostate, and Cap from 25 radical prostatectomy specimens was determined. RB immunoreactivity was present in all of the cases in the foci of HG-PIN, benign prostate, and Cap. Mutant p53 protein was detected in 56% of HG-PIN, 72% of Caps, and 20% of benign
prostatic glands. These data suggest that RB loss does not play a role in the initiation of all cases of Cap (54).

We and others have previously reported an inverse correlation between the nuclear expression levels of the RB-related gene pRb2/p130 and tumor grade or progression in different cancer types as well as a direct correlation with terminal epithelial differentiation (36, 37, 41, 42, 55–57).

Interestingly, one specimen in which there was also a HG-PIN showed the progressive loss of pRb2/p130 from normal prostatic cells to PIN cells. These data suggest that in prostate cancer, lack of expression of one or more tumor suppressor genes could be involved in the progression of the disease, from an early stage. Additionally, we have identified, using a multivariate analysis, Ki-67 and pRb2/p130 as the variables associated with a higher risk of cancer. In fact, the statistical combination of the values obtained with Ki-67 and pRb2/p130 yielded a sensitivity of 83% with a false-positive rate of 17%. An association between high proliferative rate (high Ki-67 immunoreactivity) and poor prognosis has been reported previously in prostate carcinoma (58). Our data indicate that a high Ki-67 (MIB-1) proliferative fraction and pRb2/p130 nuclear negativity could be used as prognostic markers, in addition to parameters such as histopathological tumor grade. One of the limitations of this approach is the known heterogeneity of prostate cancer. Nonetheless, in the present study, we used tissue from radical prostatectomy specimens and, therefore, had enough tissue to perform a multiple reading of the immunohistochemical stainings. Of course the ideal application of medical screening is to use the less invasive and more sensitive techniques at the time of the original diagnosis. Therefore, one would want to apply prognostic marker analysis to prostatic biopsies rather than to radical prostatectomy specimens to help determine the most appropriate therapy. We are now in the process of verifying our findings on prostate biopsy specimens and plan to extend our study to samples from patients who underwent hormone therapy and radiotherapy, also with the intent of observing possible modifications in the expression of those molecular markers that could serve as an index for assessing disease progression.

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