The Prolyl Isomerase Pin1 Is a Novel Prognostic Marker in Human Prostate Cancer

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ABSTRACT

Prostate cancer (PCa) is the most common male cancer in the United States. A major challenge that remains is to predict the clinical outcome in managing PCa patients. The prolyl isomerase Pin1 has been shown to be overexpressed in some human cancer tissues and thought to be an important player in several oncogenic pathways. However, the relationship between Pin1 expression and clinical outcome of cancer patients has not been explored. In this study, we investigated the role of Pin1 in human PCa progression and its clinicopathological significance. Immunohistochemical assessment with affinity-purified polyclonal Pin1-specific antibodies was performed on formalin-fixed paraffin sections of tissue microarray composed of 580 radical prostatectomy specimens. As determined by visual semiquantitation and confirmed by automated image analysis quantitation, Pin1 expression was positively correlated with clinical stage. Furthermore, Cox survival analysis results indicated that patients with a higher Pin1 expression had a significantly higher probability of recurrence than their counterparts with low Pin1 expression, as defined by a serum prostate-specific antigen level of ≥0.4 ng/ml on two consecutive occasions after radical prostatectomy. In addition, patients with high Pin1 expression had almost 4 times the risk of having earlier recurrence than those with low Pin1 expression; patients with a very high level had 8.1 times the risk of an earlier recurrence than a low Pin1 expressor. Pin1 was also an excellent predictor of recurrence in the subset of patients with Gleason score 6 or 7 when analyzed separately: a patient with high Pin1 expression had 8.6 times the risk of having earlier recurrence than one with low Pin1 expression. Pin1 expression is as good as or better than currently used postoperatively available clinicopathological parameters and potentially could be used in the preoperative setting to assist in choice of treatment. Thus, this study suggests a role for Pin1 expression as a potentially excellent prognostic marker in PCa and suggests that Pin1 may also serve as a novel therapeutic target for PCa.

INTRODUCTION

PCa is the most common male cancer in the United States (1–3). Although it is a very common disease, we currently have very few prognostic markers that can distinguish between tumors with a high recurrence potential and those that are not likely to recur (4). Screening asymptomatic men for the level of serum PSA has led to earlier detection of PCa and can provide an enhanced opportunity for curative surgery (5). However, the PSA level is not a reliable prognostic indicator for PCa because it does not correlate well with clinical outcome, particularly in patients with PSA values of ≥10 ng/ml (6). Survival and prognostic markers in PCa are extremely important, not only due to the mortality associated with PCa but also because of the morbidity associated with the current forms of therapy for this disease.

Several clinical features of PCa (before therapy) including clinical stage, degree of tumor cell differentiation or GS, and the serum PSA are used in routine clinical practice to separate patients into groups at low, intermediate, and high risk for tumor recurrence after local therapy (7). However, the vast majority of PCa patients are diagnosed within the PSA range of 3.0–9.9 ng/ml, with intermediate GSs of 6 or 7, and early clinical stage T1c–T2; the biological behavior of this group of patients is highly variable (7, 8). Some might have been treated unnecessarily, because the postoperative prognostic features of the tumor prove highly favorable, whereas others might have had too aggressive a disease to be cured by surgery alone. It is therefore important to seek to refine the prognostic information gained from pretreatment variables and from PCa biopsy specimens in particular.

Numerous ongoing trials are attempting to determine which patients really require a therapeutic intervention, and which ones can be followed with a careful observation. Attempts to explore genetic correlates of tumor behavior have found alterations in a number of candidate genes associated with PCa progression, including loss of p53, amplification of Myc, loss of p27, and loss of pTEN (9). In addition, recent genomic methodologies have been used to discover consistent gene expression patterns associated with a given histological or clinical phenotype in PCa (10, 11). However, no single gene has been shown to have sufficient prognostic utility to warrant clinical implementation. It remains a major challenge to predict the clinical outcome in managing PCa patients, especially those with intermediate-risk clinical features (GSs 6 and 7).

Phosphorylation of proteins on serine/threonine residues precedes proline (pSer/Thr-Pro) is a key regulatory mechanism for the control of cell proliferation and transformation (12, 13). The pSer/Thr-Pro moiety in proteins exists in two distinct cis- and trans-conformations, whose conversion is catalyzed specifically by Pin1, which specifically acts only on the phosphorylated Ser/Thr-Pro bonds (14–17). Functionally, Pin1 catalyzes the conversion of cis- and trans-conformations in proteins after phosphorylation, thereby having profound effects on their catalytic activity, dephosphorylation, protein-protein interactions, and subcellular localization. Pin1 is essential for mitotic progression and is required for the DNA replication checkpoint (14, 16, 18–27). Therefore Pin1 plays an important role in cell cycle regulation. Temperature-sensitive mutations or deletion of Ess1 (the Pin1 homologue in budding yeast) results in mitotic arrest and nuclear fragmentation (15, 28, 29). Inhibition of the Pin1 function in human tumor cells using expression of the Pin1 antisense RNA or dominant-negative mutants induces mitotic arrest and apoptosis (15, 30, 31). Depletion of Pin1 in Xenopus extracts induces premature mitotic entry and disrupts a DNA replication checkpoint (21). These results suggest that the level and function of Pin1 are pivotal for cell proliferation.

We have previously shown that Pin1 was overexpressed in some human malignancies and that its expression closely correlates with the level of cyclin D1 in human breast cancer (22, 23). Up-regulation of Pin1 has been shown to potentiate the function of several oncogenic pathways. For example, Pin1 elevates cyclin D1 gene expression by activating c-jun/AP-1 and β-catenin/TCF transcription factors (22,
Furthermore, Pin1 can bind directly to phosphorylated Thr-Pro motif in cyclin D1 and stabilize nuclear cyclin D1 protein by inhibiting its export into the cytoplasm, where it is normally degraded by ubiquitin-mediated proteolysis (24). Moreover, Pin1 is an E2F target gene that is essential for the Neu/Ras-induced transformation of mammary epithelial cells (32). These results indicate that overexpression of Pin1 plays an important role in oncogenesis. However, the relationship between Pin1 expression and the clinical outcome of cancer patients remains unknown.

The present study was undertaken to test the viability of Pin1 expression as a prognostic marker in PCa.

MATERIALS AND METHODS

Cohort Enrollment and Follow-up. As of March 2002, there was information on 6201 patients with BPH or PCa in the Baylor Medical Informatics Core Specialized Programs of Research Excellence Database. Over 3900 of these patients underwent radical prostatectomy at one of the Baylor College of Medicine-affiliated institutions and willingly provided tissues (IRB H-1158). Of these patients, 1291 were operated on between 1983 and 1998. The Baylor Institutional Review Board (IRB H-11436) approved this study. Entry criteria for this retrospective cohort study to create a radical prostatectomy tissue array included: (a) operated on by a single surgeon between 1983 and 1998; (b) no preoperative adjuvant therapy such as radiation or hormonal therapy; (c) radical prostatectomy specimen in the tissue bank; and (d) PCa present in the surgical specimen and large enough to be cored for microarrays. A total of 640 patients fulfilled the above-mentioned criteria and were cored to produce a large outcomes array.

Radical prostatectomy specimens from these patients were processed using whole mount slides according to procedures described previously (33). A single pathologist (T. M. W.) performed the pathological analysis that included staging, pathological stage, margins, capsular penetration, SVI, biopsy and prostatectomy primary and secondary GSs, LN status, tumor volume, and geographic location. The clinical and pathological data of patients who met the entry criteria were available for analysis in the Baylor Prostate Specialized Programs of Research Excellence data bank. The clinical follow-up data include PSA recurrence (defined as PSA > 0.4 ng or two consecutive rises), clinical metastasis, and death.

Tissue Microarray. Slides from all 640 radical prostatectomy specimens were reviewed and mapped. The tissue microarrays were built using a manual tissue arrayer (Beecher Instruments, Silver Spring, MD). The index tumor, defined as the largest and/or highest GS tumor, was identified on the slide, and areas representative of the index cancer were circled. Areas of normal peripheral zone away from the tumor were also circled, as well as areas of BPH. Previous studies indicate that triplicate 0.6-mm punches reliably reproduce immunohistochemical markers, even for low expression markers such as Ki67. Therefore, triplicate 0.6-mm cores were obtained from the circled areas of tumor, normal peripheral zone, and BPH and transferred onto a recipient paraffin block. To assess the adequacy of the stain throughout the sections, up to 10 different types of tissues within each 0.6-mm control core were also included in a pre-established pattern throughout each one of the blocks. A database was built for every block produced, including the coordinates of each core and the area and case of origin. The final tissue array set consisted of 15 blocks with 9 cores for every one of the 640 patients, for a grand total of approximately 6000 cores. Five-μm sections from the array blocks and tissues were cut without use of the transfer tapes.

Immunohistochemistry. Pin1 polyclonal antibodies were affinity purified using CNBr-activated Sepharose 4B column as described previously (35). Microtissue array sections were stained with affinity-purified Pin1 antibodies using a protocol described previously (22), with some modifications. Briefly, sections were deparaffinized, rehydrated, and subjected to antigen retrieval. The sections were heated in antigen retrieval citrate solution (pH 6.0; BioGenex, San Ramon, CA) with a 1300 W microwave oven (Inverter, the Genius 1300W; Panasonic) for about 2 min at a full power level. Once the solution temperature reached 100°C, the sections undergo continual heating at this level for an additional 15 min. Endogenous peroxidase activity in sections was inactivated in 3% H2O2 for 10 min. The sections were then blocked with 3% normal horse serum in 0.2 M TBS (pH 7.4) and followed by incubation in affinity-purified Pin1 antibodies (4 mg/ml) diluted at 1:10,000 in TBS overnight at 4°C. They were then processed following a standard avidin-biotin complex immunostaining procedure with an avidin-biotin complex kit (Vector Laboratories). Immunoreaction products were visualized using a 3,3′-diaminobenzidine substrate. The sections were then counterstained with hematoxylin.

To verify the specificity of the immunoreactions, some sections were incubated in either TBS or normal rabbit serum replacing for the Pin1 antibody.

Image Digitizing. Slides were first digitized at Baylor College of Medicine using an automated slide scanner (Bacus Laboratories) to produce high-resolution images used for visual semiquantitative analysis. This system also informs the dot coordinates on the slide, which permits tracking down each dot to origin and subsequent correlation with the clinical database. Slides were subsequently scanned independently at Pintex Pharmaceuticals using the ACIS (ChromaVision Medical Systems, Inc., San Juan Capistrano, CA). This system combines automated microscopy and computerized image analysis of immunostained histological sections to provide a wider range of the intensity of the Pin1 immunostaining signal and the percentage of Pin1-positive cells.

Computerized Image Analysis Assessment of Immunostaining. Positive staining (brown color) as viewed by light microscope indicates the presence of the protein, and color intensity correlates directly with protein quantity (expression). The ACIS is able to recognize 255 levels of immunohistochemical staining intensity (0–255) and convert these to fractional scores for the selected individual areas. However, because the system is very sensitive, the base limit on the threshold for the Generic 3,3′-diaminobenzidine is pre-set at 50 by the manufacturer. Therefore, any intensity values used in the analysis are the readout intensity subtracted by 50, and all measured intensity below 50, which would have to be an extremely light brown, was treated as “0” in this study. Entire immunostained tissue sections were scanned using the ×4 objective, and images were captured using the ×10 objective. In this study, we used the intensity scoring and percentage of positive scoring (the percentage of brown divided by blue + brown area) for entire tissue dot selected. The immunohistochemical staining was quantified without knowledge of the pathologist’s visual semiquantitative scores.

Statistical Analysis. Associations between clinical/pathological parameters and Pin1 expression were evaluated using Spearman correlation coefficient test. For survival analysis, the end point was the PSA biochemical recurrence. Time to recurrence was defined as the time interval between the date of surgery and the date of PSA recurrence. The predictive value of Pin1 for recurrence-free survival was evaluated using the Kaplan-Meier actuarial analysis and the log-rank test. Kaplan-Meier survival curves were constructed for patients with low, high, and very high Pin1 levels. The differences between the survival curves of these groups were tested for statistical significance using the log-rank test. The cutoff points were identified through the detailed analysis of behavior of log-rank P values across the range of the Pin1. The Cox univariate and multivariate proportional hazard models were used to determine the HRs. In the multivariate analysis, the models included the status of LNs and SMs, the presence or absence of SVI and EPE, clinical stage, GS, and preoperative PSA levels. The HR and its 95% confidence interval were recorded for each marker. P values of <0.05 were considered statistically significant in all of our analyses. Because about 10 log-rank tests were done to identify each cutoff point for the recurrence-free survival analysis, a P of <0.005 identifies significant differences that can be generalized beyond the data set used. All analyses were performed with statistical software SPSS 11.0 (SPSS Inc., Chicago, IL).

RESULTS

Clinical Characteristics. To examine whether Pin1 expression correlates with the clinical outcome of PCa patients, we analyzed Pin1 expression using tissue arrays containing 640 radical prostatectomy specimens that met three entry criteria, as described in “Materials and Methods” and “Results.” Of 640 samples, clinical characteristics and follow-up were available for 580 cases, which were then used in the subsequent study.

The patients were 87.9% Caucasians, 7.4% Hispanic, 3.5% African American, and 1.2% Asian or Middle Eastern. The age ranged from 37 to 80 years, with a mean of age of 62 years and a median age of 63 years. The patients were postoperatively followed-up for an average of 46.4 ± 33.7 months (mean ± SD; median = 43.5 months; 6245
Maximum follow-up was 153.2 months. Preoperative PSA level was available in 576 PCa cases and ranged from 0.3 to 100 ng/ml (median, 10.7 ng/ml) and a SD of 11.2 ng/ml (median, 7.3 ng/ml). Thirty percent of the patients had a preoperative PSA level ≥10.5 ng/ml. Approximately 7% had a GS 6, 85% had a GS of 6 or 7, and 8% had a higher GS (8–10). LN metastasis was found in 38 (6.4%) patients, and biochemical recurrence was seen in 117 patients (19.7%). EPE was found in 45.1% of the patients, SM positive status was seen in 15.6% of the patients, and SVI was found in 12.7% of the patients. The clinical characteristics are summarized in Table 1.

**Visual and Automated Quantitation of Pin1 Immunostaining.** Using affinity-purified polyclonal Pin1 antibody, we conducted immunohistochemical study on paraffin sections of human PCa microtissue arrays. Positive immunostaining was observed in the cytoplasm and nucleus of cancer cells. When the Pin1 antibody was preincubated with His-tagged Pin1 proteins, the immunostaining disappeared, showing the specificity of Pin1 antibody staining. Levels of Pin1 expression were heterogeneous in PCa tissues; some showed no levels (Fig. 1A), and some showed moderate levels (Fig. 1B), whereas others showed high levels of expression (Fig. 1C). Stromal cells surrounding tumors showed no Pin1 expression. After Pin1 immunohistochemistry, images were captured using the Bliss system, and Pin1 expression was evaluated visually and semiquantified by a single pathologist. The scoring system was from 0 to 3 + for both the intensity of Pin1 stain and the percentage of Pin1-positive cells (labeling frequency percentage). For the intensity, the grading scale ranged from 0 (no detectable signal), to 1 + (weak signal seen only at intermediate to high power), 2 + (moderate signal seen at low to intermediate power), and 3 + (the strongest signal seen at low power). For the percentage, the scale ranged from 0 (0%), to 1 + (1–33%), 2 + (34–66%), and 3 + (67–100%). Pin1 was expressed in 57.5% of the PCa tissues, 13.4% of the surrounding BPH, and 41.2% of the surrounding noncancerous tissues. However, high intensity levels of Pin1 expression (2 + or 3 +) were seen in 11.7% of cancers as compared with 6.5% of surrounding noncancerous tissues. The Wilcoxon signed ranks test indicates that Pin1 levels in cancer tissues were higher than those in surrounding noncancerous tissues (P = 0.0025).

To obtain an independent and more objective evaluation of Pin1 expression, Pin1 immunostaining was also quantified using the ACIS, which produced two continuous values. One is the intensity, ranging from 0 to 105, and the other is the percentage of Pin1-positive cells.

![](https://cancerres.aacrjournals.org) Fig. 1. Immunohistochemical staining of prostate cancer tissues with affinity-purified Pin1 polyclonal antibodies. A, PCa tissues showing no Pin1 staining. B, PCa tissues showing moderate levels of staining. C, PCa tissues showing high levels of staining.

<table>
<thead>
<tr>
<th>Clinical/pathological factors</th>
<th>Intensity High</th>
<th>Percentage average</th>
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<tbody>
<tr>
<td>N (%)</td>
<td>Mean (SD)</td>
<td>Mean (SD)</td>
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<td>TNM* stage</td>
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<tr>
<td>T1</td>
<td>182 (31)</td>
<td>70.7 (15.4)</td>
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<td>T2</td>
<td>356 (61)</td>
<td>71.7 (16.7)</td>
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<td>T3</td>
<td>42 (7)</td>
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<td>7</td>
<td>300 (52)</td>
<td>71.6 (16.8)</td>
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<td>8–10</td>
<td>47 (8)</td>
<td>72.6 (23.3)</td>
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<td>Extraprostatic extension</td>
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<tr>
<td>Positive</td>
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<td>72.0 (16.4)</td>
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<tr>
<td>Negative</td>
<td>322 (55)</td>
<td>71.2 (16.1)</td>
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<tr>
<td>SVI</td>
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<tr>
<td>Positive</td>
<td>72 (12)</td>
<td>73.8 (15.3)</td>
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<tr>
<td>Negative</td>
<td>508 (88)</td>
<td>71.3 (16.4)</td>
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<td>LN metastasis</td>
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<tr>
<td>Positive</td>
<td>37 (3)</td>
<td>73.3 (19.1)</td>
</tr>
<tr>
<td>Negative</td>
<td>543 (97)</td>
<td>71.5 (16.5)</td>
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<tr>
<td>SM</td>
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<tr>
<td>Positive</td>
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<td>70.3 (19.2)</td>
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<tr>
<td>Negative</td>
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<td>UICC Staging</td>
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<tr>
<td>Age</td>
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</tr>
<tr>
<td>Preoperative PSA</td>
<td>564</td>
<td>0.724</td>
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</table>

* TNM, tumor, node, metastasis. UICC, Union International Contra Cancer
When results obtained from visual and automated semiquantitative procedures were analyzed, we found a significant correlation between these two methods ($P = 0.444; P \leq 0.0001$), although correlation coefficients were not as high as might be expected. We believe that this is due to the much more discrete nature of the visual measurements and the continuous nature of the automatic imaging system measurements.

**Correlation between Pin1 Expression and Clinicopathological Variables.** Based on the automatic imaging analysis, Pin1 expression was grouped into three groups: low intensity of expression (0–23) with 364 patients; high intensity of expression (23.01–54) with 195 patients; and very high intensity of expression (≥54) with 21 patients. Pin1 expression was positively correlated with staging ($P = 0.09; P = 0.0284$). However, no correlation was found with GS, status of SM or LN, the likelihood of EPE or SVI, or the preoperative PSA level. Continuous measure of Pin1 expression was not correlated with any of these variables.

**Kaplan-Meier Survival Analysis and Cox Multivariate Regression Analysis Visual Semiquantitation.** The recurrence-free survival curves were plotted by Kaplan-Meier actuarial model. According to the results of the log-rank test, there was a significant difference in recurrence-free survival between PCa patients with any Pin1 expression and those without Pin1 expression, with those patients with no Pin1 staining having better disease-free survival as compared with those with any degree of Pin1 staining. Both the intensity and the percentage of positive cells in the tumor were predictive of time to recurrence on univariate as well as multivariate analyses. The presence of the Pin1 stain was a significant predictor of time to recurrence on univariate analysis (HR = 1.60; $P = 0.0198$) as well as on multivariate analysis that adjusts for other clinicopathological parameters (HR = 1.75; $P = 0.0105$; Fig. 3). However, visual semiquantitation was not able to distinguish survival differences among different levels of Pin1 staining intensity.

The percentage of Pin1-positive tumor cells was also a significant continuous predictor (HR = 1.004; $P = 0.0343$) of time to recurrence. As the result of the optimal cutoff chosen at 10%, the division of patients completely agreed with the intensity method described above and produced identical results on univariate and multivariate analyses. Finally, the sum index (intensity + percentage) was a significant continuous predictor (HR = 1.004; $P = 0.0343$) of time to recurrence. Patients were then stratified into “0” and “>0” groups, resulting in groups identical to those in the intensity and percentage methods and, therefore, in identical results on univariate and multivariate analyses.

**Automated Image Analysis.** The presence and degree of Pin1 expression in the surrounding noncancerous prostate tissue or BPH were not significant predictors of biochemical recurrence-free survival (data not shown). However, both the intensity of the Pin1 staining (HR = 1.01818; $P = 0.0024$) and percentage of Pin1-positive cells ($P = 0.008875$; HR = 1.037; $P = 0.0075$; (1.010 03-1.06420) in PCa tissues were significant continuous predictors of time to biochemical recurrence.

In addition, the intensity of the Pin1 staining was inversely associated with the recurrence-free survival; higher-level expression of Pin1 was a significant predictor of earlier recurrence. After an extensive search for the optimal cutoff, three groups were identified: (a) low intensity of expression (0–23) with 364 patients; (b) high intensity of expression (23.01–54) with 195 patients; and (c) very high intensity of expression (≥54) with 21 patients. Differences between groups were significant on univariate as well as multivariate analyses (Fig. 4). Biochemical recurrence-free survival decreases with increased intensity levels. Of two patients with otherwise identical clinicopathological parameters, a patient with high Pin1 intensity levels had almost twice the risk of having earlier recurrence than the one with low Pin1 levels, a patient with very high Pin1 intensity levels had almost 2.5 times the risk of having earlier recurrence than one ranging from 0 to 99.8 (Fig. 2). Because of the triplicate nature of the arrays, three values of both the intensity and percentage were obtained from every patient. To represent the intensity hot spot, the highest intensity value was used for the intensity analysis, whereas the average of the three percentage values was considered for the percentage analysis. In addition, because our initial analyses suggested that both the highest intensity and the average percentage value of Pin1 staining were important, they were also added together to generate the sum index. The mean intensity value of PCa was 21.9, as compared with 17.4 for surrounding noncancerous tissues and 13.0 for BPH. The Wilcoxon signed ranks test again indicated that Pin1 levels in cancer tissues were higher than those in surrounding noncancerous tissues ($P = 0.0001$).
with high Pin1 levels, and a patient with a very high level had more than 6.8 times the risk of a low-level patient. Furthermore, based on multivariate analyses, the Pin1 intensity was an independent prognostic marker that had significantly higher HR in predicting disease-free survival than other commonly used clinical or pathological features.

An even stronger relationship between Pin1 and clinical outcome was found when the Pin1 sum index (intensity + percentage) was used in the analyses (Fig. 5). The sum index was significant as a continuous predictor with high levels of Pin1 expression associated with decreased biochemical free survival (HR = 1.015; \( P = 0.0010 \)).

After determining the best cutoff points, three groups were again identified: (a) low-level expression (0–74) group had 375 patients; (b) high-level expression (74.01–120) group had 189 patients; and (c) very high level expression (>120) group had 16 patients. Differences among these groups were again significant on univariate and multivariate analyses (Fig. 5). Of two patients with otherwise identical clinicopathological parameters, a patient with high Pin1 sum index had almost 1.9 times the risk of having earlier recurrence than one...
with low Pin1 levels, a patient with very high Pin1 sum index had almost 4.3 times the risk of having earlier recurrence than one with high Pin1 levels, and a patient with very high level expression had more than 8.1 times the risk of a patient with low-level expression. These results indicate that Pin1 level is an excellent novel prognostic marker in PCa patients, with high-level expression of Pin1 having a much shorter biochemical recurrence-free survival time.

To compare Pin1 against currently used markers, we first grouped patients into very high Pin1 index (>120) and low + high Pin1 index (≤120) categories, very high PSA (>10) and low + high PSA (≤10) categories, and very high GS (>7) and low + high GS (≤7) categories. Postoperatively, multivariate Cox models suggest that Pin1 index [HR = 9.1 (4.4–18.8); P < 0.0001] is a significantly better postoperative marker for selecting very high-risk patients than PSA [HR = 2.4 (1.6–3.8); P < 0.0001] and GS [HR = 2.7 (1.7–4.3); P = 0.0001] and an as good or better marker than LN metastasis [HR = 3.2 (1.9–5.1); P < 0.0001], ECE [HR = 3.0 (1.7–5.2); P = 0.0001], SVI [HR = 3.4 (2.2–5.2); P < 0.0001], and SMs [HR = 3.3 (2.2–5.0); P < 0.0001]. Similar analysis, repeated for low versus high + very high categories (Pin1 index, ≤74 and >74; PSA, ≤4 and >4; GS, ≤6 and >6) showed that Pin1 is as good a marker for distinguishing low risk patients as other markers currently used.

**GS 6 and 7 Patients.** Because the majority of PCa patients who undergo prostatectomy fall within this category (having intermediate risk clinical features) and also because of limitations in our prognostic capacity, we examined this subgroup separately. After determining the best cutoff point, patients were grouped into high Pin1 index (>122) and low Pin1 index (≤122) categories and into high PSA (>10) and low PSA (≤10) categories. Postoperatively, multivariate Cox models suggest that Pin1 index [HR = 9.3 (3.7–23.1); P < 0.0001] is a significantly better postoperative marker than PSA [HR = 1.9 (1.1–3.1); P = 0.0127] and as an as good or better marker than LN metastasis [HR = 4.3 (2.4–7.8); P < 0.0001], ECE [HR = 2.9 (1.6–5.5); P = 0.0008], SVI [HR = 2.9 (1.7–5.0); P = 0.0001], and SMs [HR = 4.2 (2.6–6.8); P < 0.0001; Fig. 6]. Of two patients with otherwise identical clinicopathological parameters, a GS 6 or 7 patient with a Pin1 index > 122 has more than 6 times the risk of having earlier recurrence than one with low Pin1 levels. These results indicate that Pin1 is an excellent new predictor of recurrence in patients with GS 6 and 7.

**DISCUSSION**

Pin1 has been shown to play an important role in oncogenesis. However, it is not known whether there is any relationship between Pin1 expression and the clinical outcome of cancer patients. To address this question, we here determined Pin1 expression in 580 PCa specimens obtained from radical prostatectomy using Pin1 immunohistochemistry, followed by both visual semiquantitative as well as automatic imaging quantitative procedures. When comparing Pin1 and clinical features, we found that Pin1 expression is positively correlated only with clinical staging, but not with other commonly used clinical features. More importantly, the Pin1 level is also a predictor of recurrence, with patients with a higher Pin1 having a significantly lower probability of recurrence-free survival than their counterparts. Moreover, the Pin1 level is also a predictor of recurrence even in patients with GS 6 and 7, where it is very difficult to predict the outcome. Finally, Pin1 level is an independent prognostic marker that has a significantly higher value in predicting disease-free survival than those of other commonly used clinical features. Thus, our study establishes the role of Pin1 as a prognostic marker for biochemical recurrence in PCa and suggests that Pin1 may be a novel target for treating the tumor.

The data presented here establish Pin1 as an independent prognostic marker for biochemical failure in PCa patients after radical prostatectomy. We have demonstrated that Pin1 is overexpressed in PCa cells compared with normal prostate tissues, both in the intensity of expression and in the percentage of Pin1-positive cells. Furthermore, the elevated Pin1 expression correlates with clinical staging. More importantly, Pin1 is a predictive marker, such that visual semiquantitation can discriminate between on-off expression and its survival significance. The expression of Pin1 by a minority of PCa cells is a strong independent predictor of biochemical recurrence in patients with PCa. Even more significant is when automated imaging system is used to evaluate Pin1 expression. Automated imaging analysis
produces results that are consistent with data obtained from visual analysis, but with a greater discriminatory power.

Automated imaging analysis discriminates even further particularly among the Pin1 intensity as well as sum index of Pin1 expression. To correct for the uncertainty of the linear intensity of expression, we also used Pin1 expression sum index, which is the sum of the highest intensity of Pin1 staining and the average percentage of Pin1-positive cells. It is noteworthy that a patient with a Pin1 high sum index has >8 times greater risk of recurrence than a patient with a low expression index. Moreover, Pin1 outperforms other known and currently used clinicopathological parameters, including LN metastasis, preoperative PSA levels, GS, SMs, seminal vesicle status, and extracapsular extension. Furthermore, our data show that Pin1 also outperforms other clinicopathological parameters when analyzing patients with GS 6 and 7 patients. Given that this category comprises the vast majority of PCa patients, and that there are no known reliable markers available to predict their clinical outcome, we believe that Pin1 has a great potential to become a novel prognostic marker in PCa.

Radical prostatectomy is a definitive form of therapy for clinically localized PCa (36). However, approximately a third of the patients treated with radical prostatectomy experience progression even when tumors are confined pathologically to the prostate (37). It is well known that patients with the same pathological disease stage and/or grade show different prognoses. Accurate prediction of the risk of recurrence would be useful when considering watchful waiting or early adjuvant therapy and some form of investigational treatment. In the preoperative setting, current standard of care relies on PSA values as well as biopsy GS for clinical decision making. To our knowledge, there is no validated prognostic marker that is able to reliably distinguish between groups of patients who could be safely entered into watchful waiting (deferred treatment) protocol versus patients that need and would benefit from definitive surgery/radiation therapy. Thus, a substantial part of PCa research aims to define accurate prognostic markers for estimating malignant potential. Because our data suggested that Pin1 was a strong independent predictor of biochemical recurrence of PCa based on the radical prostatectomy specimens, it is worth investigating whether Pin1 levels in needle biopsy samples will correlate with the clinical outcome of PCa patients.

The strong relationship between the Pin1 level and the clinical outcome of PCa suggests the involvement of Pin1 in progression of the disease. Indeed, we have previously shown that Pin1 expression is activated by oncogenic pathways via the transcriptional factor E2F and that Pin1 overexpression activates multiple steps in oncogenic signaling pathways. For example, in breast cancer, Pin1 collaborates with Ras/c-Jun NH2-terminal kinase signaling to increase the transcriptional activity of c-Jun toward cyclin D1 (22). Furthermore, Pin1 also activates β-catenin, which can induce the transcription of cyclin D1, c-Jun, and c-Myc (23, 38–41). Moreover, Pin1 can directly bind and stabilize cyclin D1 (24). Consistent with a critical role of Pin1 in the regulation of cyclin D1 function, Pin1 knockout mice have decreased cyclin D1 levels and exhibit a series of proliferative abnormalities (24), resembling cyclin D1 knockout mice (28, 29). Overexpression of Pin1 can not only confer transforming properties on normal mammary epithelial cells but can also enhance the transformed phenotypes of Neu/Ras-transformed mammary epithelial cells. In contrast, inhibition of Pin1 suppresses Neu- and Ras-induced transformed phenotypes, which can be fully rescued by overexpression of a constitutively active cyclin D1 mutant that is refractory to the Pin1 inhibition. Therefore, by isomerizing phosphorylated Ser/Thr-Pro motifs, the common phosphorylation sites in oncogenic pathways, Pin1 may function as a potent catalyst that amplifies and translates multiple oncogenic signaling mechanisms.

Elevation of Pin1 level in tumors is not uncommon because we examined 60 different types of human tumors and found that 38 of them showed overexpression of Pin1.5 It indicates that Pin1 is involved in tumor progression and that Pin1 could become a potential therapeutic target in patients with biologically aggressive tumors. Indeed, several other lines of evidence also support that inhibition of Pin1 may offer an attractive option for anticancer therapy. As described above, Pin1 is involved in activation of multiple oncogenic pathways and plays an essential role in cell transformation, at least in that induced by oncogenes Ras and Neu (22, 23, 32). Furthermore, Pin1 is an enzyme with extrav-
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dinarily high substrate specificity and a well-defined active site (16–18, 42, 43). Historically, it has been much easier to develop inhibitors specific for an enzyme such as Pin1 than for a nonenzymatic protein. Moreover, depletion of Pin1 using antisense Pin1 or dominant-negative Pin1 causes cancer cells into apoptosis in transient transfection experiments (15, 46). In addition, depletion of Pin1 also suppresses the transformed phenotypes induced by Ras/Neu in stable transfection experiments (32). Finally, because Pin1 knockout mice do reach adulthood despite some cell proliferative abnormalities, especially in old age (24, 45), it is reasonable to assume that an anti-Pin1 therapy might not have general toxic effects. However, the feasibility of therapeutic Pin1 inhibition has not yet been explored due to the lack of Pin1-specific inhibitors. In summary, the results in this study have demonstrated that Pin1 expression in PCa is an independent prognostic marker that outperforms other known and currently used clinicopathological parameters. Furthermore, even with GS 6 and 7 patients, Pin1 also outperforms other clinicopathological parameters in predicting disease-free survival. This is especially exciting because the vast majority of newly diagnosed patients are within this category, and also because currently there is not a reliable marker to predict their outcome. If these results are corroborated independently, we suggest that visual observation of Pin1 staining, coupled with the enhanced discriminatory power of measuring Pin1 expression by the automated image analysis system, may be used to discriminate recurrence in patients who have undergone radical prostatectomy. Furthermore, because tissue microarray cores have similar amounts of tissue as compared with clinical biopsies, it may be possible to detect Pin1 expression in pretherapy biopsies. If so, Pin1 could be used to triage patients into watchful waiting with greater certainty or to distinguish patients who will fail classical surgical therapies and would therefore benefit from adjuvant therapy.

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The Prolyl Isomerase Pin1 Is a Novel Prognostic Marker in Human Prostate Cancer

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