Elevated Skp2 Protein Expression in Human Prostate Cancer: Association with Loss of the Cyclin-dependent Kinase Inhibitor p27 and PTEN and with Reduced Recurrence-free Survival[1]

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ABSTRACT

The F-box protein Skp2 (Fbl1) is a positive regulator of G_1-S transition and promotes ubiquitin-mediated proteolysis of the cyclin-dependent kinase inhibitor p27. Its overexpression has been implicated in cell transformation and oncogenesis in both *in vitro* and *in vivo* models. In this study, we investigated its role in human prostate cancer progression. Immunohistochemical analysis was performed on formalin-fixed paraffin sections of 622 radical prostatectomy specimens, 74 prostatic intraepithelial neoplasm specimens, as well as in 4 normal prostate organ donors assembled into tissue microarrays. We found that both luminal and basal epithelial cells in normal prostate had very low Skp2 levels, but Skp2 levels and labeling frequency increased dramatically in both premalignant lesions of prostatic intraepithelial neoplasm ($P = 0.0252$) and in prostate cancer ($P = 0.0037$). The Skp2 labeling frequency in cancer was positively correlated with preoperative serum prostate-specific antigen level ($P = 0.0499$) and Gleason score ($P = 0.0002$), whereas the Skp2 index was positively correlated with extraprostatic extension ($P = 0.0454$), clinical stage ($P = 0.0170$), as well as Gleason score ($P = 0.0002$). Kaplan-Meier analysis revealed that a higher Skp2 labeling index (>10) was a significant predictor of shorter biochemical recurrence-free survival time after radical prostatectomy ($P < 0.0363$, log-rank test). An inverse correlation of Skp2 was observed with both its biochemical target p27 expression in prostate cancer ($P = 0.0003$) and with its putative negative regulator, the PTEN tumor suppressor protein ($P = 0.0444$). These data suggest that induction of Skp2 may be causally linked with decreased levels of p27 in prostate cancer and implicate PTEN in the regulation of Skp2 expression *in vivo*, as previous tissue culture experiments have suggested.

INTRODUCTION

Progression through the mammalian cell cycle requires the activities of Cdks,[3] composed of catalytic Cdk subunits and regulatory cyclin subunits. In particular, Cdk2 in complexes with A and E-type cyclins are critical to progression through the G_1-S transition (reviewed in Refs. 1–3). These enzymes are opposed by a family of Cdk inhibitors typified by p27^{Kip1} (reviewed in Ref. 4). p27 is expressed at its highest levels in G_0 and G_1 cells, and its levels decrease as cells enter the cell cycle. Several lines of evidence indicate that p27, together with its related family members p21 and p57, functions to limit the proliferative capacity of cells *in vivo*. Mice lacking p27 display defects in the cell cycle exit, resulting in organ overgrowth in tissues that express the highest levels of p27, pituitary hyperplasia, and in defects in differentiation (5–8). In addition, p27 has been reported to be a haploinsufficient tumor suppressor in mice (9). Although p27 is rarely mutated in human cancer, recent studies indicate that its expression is frequently lost during transformation of multiple tissues, including breast, colon, ovarian, and prostate, where p27 loss provides independent prognostic information (10–18).

p27 levels are regulated by ubiquitin-mediated proteolysis (19) in a process that requires its association with cyclin/Cdk complexes (20, 21) and phosphorylation on T187 (22). Ubiquitination of p27 involves a E3 ubiquitin ligase, SCF^{Skp2}, composed of Skp1, Cul1, Rbx1, and the F-box protein Skp2. Skp2 serves as the specificity component for this E3 and binds to p27 in a manner that depends on p27 phosphorylation and on the cofactor Cks1 (23–27). Loss of Skp2 in mice leads to increased levels of p27, consistent with a role for Skp2 in p27 turnover (28). Skp2 levels are cell cycle regulated, and it accumulates during S-phase. Inappropriate expression of Skp2 in G_0 cells can promote S-phase entry concomitant with loss of p27 (24). Skp2 is also found in complexes with cyclin A/Cdk2 in transformed cells (29), although the significance of this complex is unknown.

[3] The abbreviations used are: Cdk, cyclin-dependent kinase; PIN, prostatic intraepithelial neoplasm; PSA, prostate-specific antigen; HGPIN, high-grade PIN; ABC, avidin-biotin complex; LI, labeling index; ECE, extraprostatic extension.

A complex relationship also exists between p27 and the tumor suppressor protein PTEN. Loss of PTEN predisposes individuals to transformation in a wide variety of tissues (30). Interestingly, loss of p27 in PTEN+/− mice greatly enhances the transformation frequency in multiple lineages (31). This collaboration is most pronounced in the prostate, where transformation is fully penetrant. Interestingly, *in vitro* studies indicate that loss of PTEN leads to increased levels of Skp2 through an unknown mechanism (32).

Decreased expression of p27 is frequently observed in human prostate cancer, but the biological basis of this is not known (14, 16, 18). In this study, we have examined the relationship between p27, Skp2, and PTEN expression in human prostate cancer using tissue microarrays. We find that Skp2 levels are dramatically increased in prostate cancer, and this correlates with loss of p27 and with loss of PTEN. Increased levels of Skp2 can be seen in premalignant lesions referred to as PIN, suggesting that Skp2 induction is an early event in transformation. Skp2 levels were also inversely correlated with recurrence-free survival, suggesting that Skp2 may serve as a useful clinical marker. During the course of this work, other studies linking increased Skp2 to reduced p27 levels in colon (33), lymphoid (34), and oral epithelial tumors (35) were reported. Our data, together with *in vitro* data (32), suggest a model in which loss of PTEN in the prostate promotes destruction of p27 by allowing the accumulation of Skp2.

MATERIALS AND METHODS

Patients and Prostate Specimens. A total of 622 prostate cancer patients who had undergone radical prostatectomy were included in this study. No patient received any treatment for cancer before surgery, which were done by a single surgeon between 1983 and 1998. Institutional Review Board-approved consent forms were used to obtain consent from the patients. Preoperational information on the patient's age, blood PSA levels, clinical staging, and pathological biopsy diagnosis were recorded in the Baylor Prostate SPORE database. After surgery, the prostate specimens were sliced into 5 mm-thick tissue whole mount according to a procedure described previously (36). The tissue slices were then fixed in 10% neutral buffered formalin and embedded in paraffin. H&E-stained whole mount sections from each specimen were evaluated by a single pathologist (T. M. W.). Postoperative pathological findings including status of extraprostatic extension, seminal vesicle invasion, lymph node metastases, surgical margin, and Gleason score, together with clinical follow-up information, was also collected into the database. The patients were postoperatively followed-up for an average of 42.7 months (median, 40.6 months; range, 0.36–167.74 months), and disease recurrence was determined by a serum PSA level higher than 0.4 ng/ml on two successive measurements (Hybritech, Inc., San Diego, CA).

Array Construction and Tissue Sections. For each specimen, the index tumor was defined as the most clinically significant cancer focus (the largest and/or most invasive and/or highest Gleason score cancer focus that was identified and mapped on whole-mount sections). Nearly always, the index cancer was the largest tumor focus with the highest Gleason score and most invasive with respect to the capsule and/or

seminal vesicle. However, in rare cases, the Gleason score of the index cancer focus was less than other foci of prostate, which were judged less significant for potential clinical impact. The Gleason score of the index cancer was used as the overall surgical Gleason score of the prostatectomy and used in the analysis correlating tumor histological differentiation with protein expression. Two-mm cores were punched from tissue slices and transferred to a recipient block. The tissue microarrays were built using a manual tissue arrayer (Beecher Instruments, Silver Spring, MD). Internal controls were placed in a preestablished pattern throughout each array to assess adequacy of the stain throughout the sections. A database was built for every block produced, including the coordinates of each core and the area and case of origin. For the study of Skp2 expression in HGPIN, a second set of arrays was built using 63 radical prostatectomy specimens in which 2-mm cores were obtained from areas of HGPIN. Additionally, 11 biopsy tissues from patients who had developed HGPIN but no detectable cancer were included in this study as well. Normal prostates from 4 organ donors were used as control tissues. Five-μm sections from the array blocks and tissues were cut for immunostaining.

Immunohistochemistry. Sections were deparaffinized and rehydrated. They were then heated in citrate buffer (0.01 M, pH 8.0) in an 800 W microwave oven for 12 min for antigen retrieval. Endogenous peroxidase in sections was inactivated in 2% H_2O_2 for 10 min. The sections were then blocked in 3% normal horse serum in 0.2 M PBS (pH 7.4) and followed by incubation with a mouse monoclonal antibody to Skp2 (33), generously provided by M. Pagano (New York University, New York, NY) or a rabbit anti-PTEN antibody from Zymed. Anti-Skp2 was used at a dilution of 1:100 in PBS with 0.5% normal horse serum. Sections were incubated in primary antibody for 2 h at room temperature. They were then processed following a standard ABC immunostaining (Vector Laboratory, Burlingame, CA). Immunoreactive products were visualized using 3,3′-diaminobenzidine/H_2O_2. To verify the specificity of the immunoreactions, some sections were incubated with either PBS or normal mouse IgG replacing Skp2 antibody. Some sections were also immunolabeled with a monoclonal p27 antibody (diluted at 1:100; kindly provided by Dr. Xin Lu (Ludwig Institute, Imperial College, London, UK); Ref. 18) or with anti-Ki67 (diluted at 1:200; Immunotech, Inc.). Additionally, polyclonal antibody to cyclin A (37) diluted at 1:1000 was also used to stain some sections following a standard ABC procedure.

Quantitation of Immunohistochemistry. Nuclear Skp2 immunostaining in cancer was evaluated microscopically and recorded as the percentage of Skp2-positive cells (labeling frequency %) as well as labeling intensity scored as 0–3, where 0 was defined as lack of staining, 1 weak but distinct staining, 2 moderate staining, and 3 intense staining seen at low power. A LI defined as the function of labeling frequency and the labeling intensity was determined for each cancer specimen. p27 and cyclin A immunostaining in cancer was quantified as labeling frequency (%) as described (18). All specimens were evaluated without any knowledge of the patients' clinical information.

Statistical Analysis. The differences in Skp2 labeling frequencies were compared between normal prostate, HGPIN,
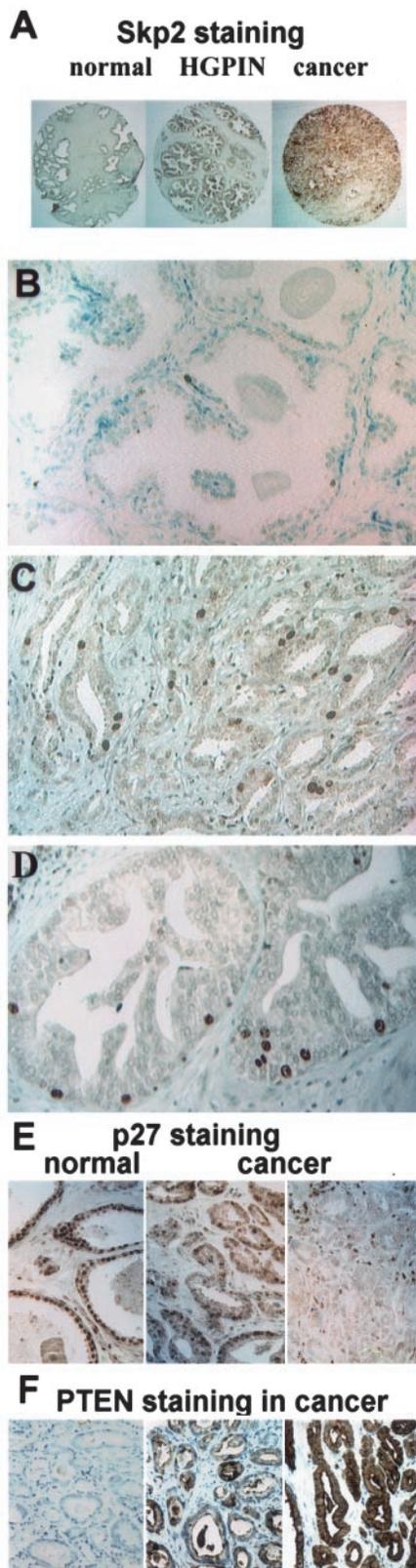
and cancer specimens by using the Mann-Whitney test. The relationship of Skp2 expression with patients' clinical and pathological variables was evaluated using the Spearman correlation coefficient. The Spearman correlation coefficient testing was also used to determine the relationship of Skp2 with PTEN and p27 levels. The significance of Skp2 and cyclin A as predictors for recurrence-free survival time was determined using the Kaplan-Meier actuarial analysis and the log-rank test. In addition, the Cox proportional hazard regression model was used to calculate hazard ratios for all univariate analyses of recurrence-free survival time markers and to determine whether Skp2 is an independent predictor to time to recurrence in the presence of other pathological and clinical markers. The hazard ratio and its 95% confidence interval were recorded for each marker. $P < 0.05$ was considered statistically significant in all of our analyses. All analyses were performed using SPSS 11.0 statistical software (SPSS, Inc., Chicago, IL).

RESULTS

Expression of Skp2 in Human Prostatic Tissues. We and others have found that loss of p27 protein levels is frequently associated with transformation. As it became apparent that Skp2/Fbl1 was associated with p27 ubiquitination (23–27), we sought to determine whether Skp2 might be up-regulated during prostate transformation. To facilitate this analysis, we used tissue microarrays (Fig. 1*A*) containing specimens from >600 individuals including 4 normal prostates from organ donors, 622 prostate cancers, as well as 74 HGPIN lesions (Fig. 1; Table 1). The Skp2 immunoreactivity was predominantly localized to the nuclei of prostatic cells (Fig. 1). Skp2 positive stromal cells were rarely seen. In 5% of the cancer specimens examined, a weak or moderate cytoplasmic immunoreactivity could be seen in addition to predominant nuclear reactivity in cancer cells.

Skp2 Expression Is Significantly Increased in Cancer Cells as Well as in HGPIN Lesions Relative to Normal Glandular Epithelium. Quantitative immunostaining data including the percentage of incidence of Skp2-positive specimens among each specimen group and cell labeling frequency (%) is summarized in Table 1. In normal prostate, Skp2 immunoreactivity was rare. Positive nuclear staining was seen only in a small proportion of glandular epithelial cells because most cells were completely negative for antibody reactivity (Fig. 1*B*). Infrequent Skp2 expression was also seen in histologically normal prostatic epithelium adjacent to cancer in array specimens (15 of 65 cases). The expression was weak and most commonly associated with atrophy. Basal epithelial cells were infrequently positive. In contrast to normal prostatic epithelium, increased Skp2 levels

Fig. 1 Skp2 expression is induced in prostate cancer and PIN lesions in a tissue microarray. *A,* low-power image of a tissue microarray stained with anti-Skp2 antibodies. Skp2 protein expression as demonstrated by ABC immunohistochemical staining in normal prostatic tissue (*B*), HGPIN (*C*), and prostate cancer (*D*). Skp2-positive cells were rarely seen in normal glandular epithelium (*B*). In contrast, increased levels of nuclear Skp2 was seen in both cancer and HGPIN specimens (*C* and *D*). *E* and *F,* tissue microarray sections were stained using either anti-p27 antibodies (*E*) or anti-PTEN antibodies (*F*). In *F,* tumor sections displaying low (*left panel*), moderate (*middle panel*), and high (*right panel*) levels of PTEN staining are shown.

Table 1 Skp2 protein expression in human prostatic tissues[a]

Tissue	n[b]	Skp2 labeling frequency (%)			
		Median	Mean ± SE	Incidence	%
Normal prostate	4	0.50	0.50 ± 0.29	2 of 4	50.0
HGPIN lesions	74	10.00[c]	21.30 ± 2.83	64 of 74	86.4
Cancer-associated HGPIN only	63	10.00	21.68 ± 3.17	57 of 63	90.4
Biopsies	11	20.00	19.09 ± 5.95	7 of 11	63.6
Primary tumors	622	30.00[d]	36.32 ± 1.22	557 of 622	89.2

[a] Nuclear Skp2 immunostaining was demonstrated in normal prostatic epithelial cells from tissue donors, HGPIN lesions (with or without cancer), as well as in overt cancers.

[b] Number of specimens.

[c] Frequency was significantly higher than in normal prostate tissue ($P = 0.0252$).

[d] Significantly higher than both normal prostate tissue and HGPIN lesions ($P = 0.0037$ or $P < 0.0001$, respectively).

Table 2 Skp2 expression in 622 human prostate cancers[a]

	n[b]	Skp2 labeling frequency (%)	
		Median	Mean ± SE
Patients	622	30.0	36.32	1.22
Preoperative PSA (ng/ml)				
<4	94	15.0	28.79	2.98
4–10	325	30.0	37.96	1.70
10–20	112	30.0	35.00	2.83
>20	72	45.0	44.44	3.54
Clinical stage (UICC[c] system)				
T_1	216	30.0	34.56	2.03
T_2	359	30.0	35.62	1.63
T_3	43	60.0	48.72	4.36
Gleason score				
3–6	277	20.0	31.96	1.79
7	304	35.0	39.26	1.76
8–10	41	50.0	43.93	4.77
ECE				
Yes	263	30.0	38.22	1.88
No	359	30.0	34.92	1.61
Margins positive				
Yes	88	30.0	35.07	3.27
No	534	30.0	36.52	1.32
Seminal vesicle involvement				
Yes	83	30.0	38.57	3.03
No	539	30.0	35.97	1.33
Lymph node involvement				
Yes	42	55.0	44.52	4.54
No	580	30.0	35.72	1.27

[a] Skp2 levels expressed as nuclear labeling frequency of cancer cells in tissue microarray composed of 622 radical prostatectomy specimens stratified according to their pathological and clinical features.

[b] Number of specimens.

[c] UICC, Union International Centre Cancer.

were demonstrated in cancer specimens (Fig. 1*C*). Skp2 was detected in 557 of 622 (89.2%) patients, and the Skp2 labeling frequency in cancer [mean, 36.32; median, 30] was significantly higher than either normal prostate epithelium [mean, 0.50; median, 0.50; $n = 4$; $P = 0.0037$, Mann-Whitney test] or the HGPIN (mean, 21.30; median, 10.00; $n = 74$; $P < 0.0001$, Mann-Whitney test; Table 1). In specimens containing HGPIN lesions, increased Skp2 expression, relative to the normal epithelium, was also seen (Fig. 1*D*). In HGPIN, the Skp2 labeling frequency was significantly higher than that found in the normal glandular epithelia ($P = 0.0252$; Mann-Whitney test).

Increased Skp2 Protein Levels Are Associated with Poor Histological Differentiation of Prostate Cancer. Relationships of Skp2 levels with pathological and clinical variables including ECE, seminal vesicle invasion, surgical margin, positive lymph node, Gleason score, preoperative PSA levels, as well as clinical stage were evaluated (Table 2). Although not statistically significant, cancer with positive lymph node deposits, ECE, positive seminal vesicle invasion, or a higher clinical stage appeared to have a higher Skp2 labeling frequency (Table 2). However, ECE and clinical stage were significantly correlated with the Skp2 LI (Table 3). A significant correlation was found between the Skp2 labeling frequency and the Gleason score in radical prostatectomy specimens (rho = 0.150; $P = 0.0002$; Table 3). Cancers with higher Gleason scores tended to have a higher level of Skp2 expression (Fig. 2). Preoperative PSA levels were also positively correlated with Skp2 frequency (rho = 0.80; $P = 0.0499$; Table 3).

Previous studies have indicated that Skp2 expression is cell cycle regulated, and in principle, increased frequency of Skp2 staining could simply represent proliferation as opposed to increased expression independent of proliferation. If this were the case, we would expect rates of Ki-67 staining to parallel those of Skp2. When patients were stratified according to the Skp2 labeling index, we found that patients with a Skp2 LI >10 ($n = 52$) had a significantly higher labeling frequency of Ki-67 (mean, 3.77; median, 2.95) than that found with patients displaying a Skp2 LI ≤10 ($n = 36$; mean, 2.54; median, 2.12; Mann-Whitney test, $P = 0.0408$). These results indicate that cancers with a higher Skp2 level tend to display higher proliferation rates. However, it should be noted that the cancers with high-level Skp2 expression had a broad range of Ki-67 labeling indices (from 0.31 to 16.25%). The Ki-67 labeling index was not significantly correlated with the continuous Skp2 labeling frequency (Spearman's rho = 0.197; $P = 0.0658$) or the continuous Skp2 labeling index (Spearman's rho = 0.155; $P = 0.1494$). Thus, Skp2 expression is not simply linked to proliferation in prostate cancer tissues.

Increased Skp2 Levels Predict a Shorter Recurrence-free Survival Time. Further analysis was performed to determine the value of using Skp2 expression as a predictive marker for patients' recurrence-free survival. Of 622 patients examined, 616 had follow-up data for this analysis. These patients were followed up after radical prostatectomy for a time ranging from

Table 3 Correlation of Skp2 with clinical and pathological parameters of prostate cancer

Skp2 *versus*	n	Labeling frequency (%)		LI	
		rho[a]	P	rho[a]	P
Gleason score[b]	622	0.150	0.0002	0.148	0.0002
Positive surgical margins	622	−0.020	0.6201	−0.005	0.8928
ECE	622	0.049	0.2222	0.080	0.0454
Seminal vesicle involvement	622	0.046	0.2561	0.067	0.0926
Clinical stage	618	0.076	0.0585	0.096	0.0170
Preoperative PSA (ng/ml)	603	0.080	0.0499	0.073	0.0746
Positive lymph node	622	0.068	0.0916	0.076	0.0590

[a] Spearman correlation coefficient.
[b] Radical prostatectomy specimens.
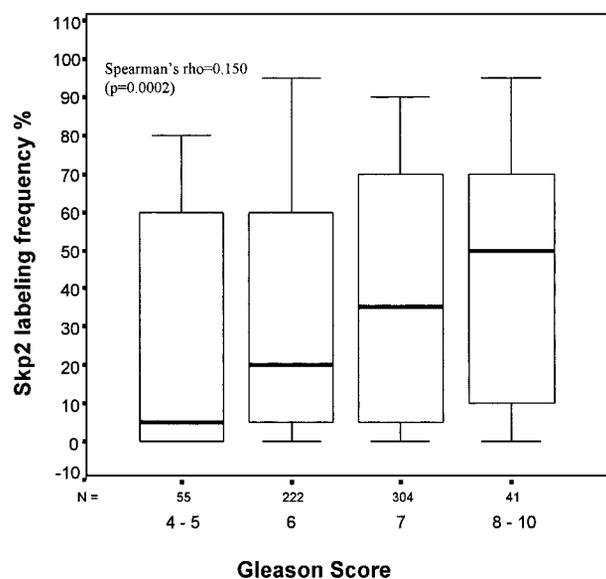

Gleason Score

Fig. 2 Correlation of Skp2 protein levels and cancer histological differentiation. Skp2 labeling frequency were positively correlated with Gleason scores of cancers in radical prostatectomy specimens (rho = 0.15; $P = 0.0002$, Spearman correlation coefficient testing). For each Gleason group, the box indicates the values of middle 50% of data. The dark line within a box denotes the median and the bars indicate the range of data.

Months of follow-up

Fig. 3 Increased Skp2 expression in prostate cancer as a predictor of time to recurrence. A comparison between patients with a Skp2 LI ≤10 and their counterparts with an index of >10 is shown.

0.36 to 167.74 months (mean, 42.7; median, 40.6 months). One hundred twenty-two had biological recurrence (a blood PSA level ≥0.4 ng/ml) during follow-up. A lower quartile of the Skp2 labeling index was used as the breakpoint between relatively low *versus* high Skp2, creating subgroups at 10. At this cutoff point, 222 cases were in the low category (LI, ≤10). Three hundred and ninety-four patients had an index >10 (LI, >10).

As shown on the Kaplan-Meier plot (Fig. 3), the probability of recurrence-free survival in the high LI group was significantly lower than that in the low LI group ($P = 0.0363$; Log-rank test). Using the Cox proportional hazard model, we showed that patients with a high Skp2 LI (>10) were 50% more likely to have an earlier recurrence than patients with low Skp2 labeling index. Other pathological markers were analyzed univariately for predictive value. As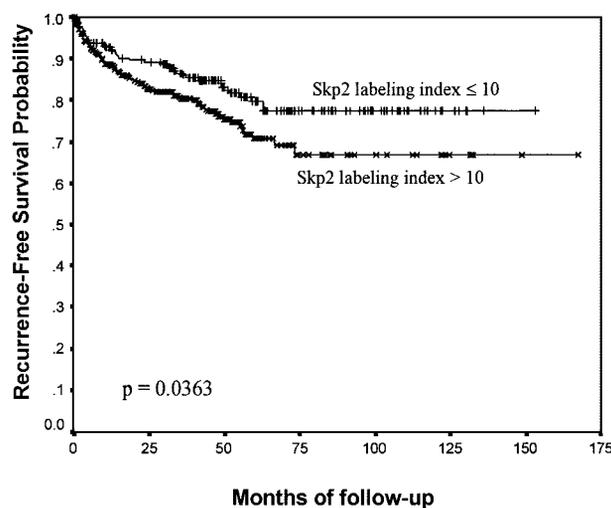 expected, ECE, seminal vesicle invasion, lymph node metastasis, surgical margin status, and Gleason score were all significant predictors of time to recurrence (Table 4). However, the Skp2 labeling index was not an independent predictor of other pathological markers of recurrence-free survival time based on multivariate analysis ($P = 0.8724$).

Skp2 Expression Inversely Correlates with p27 and PTEN Levels in Cancer. Given the biochemical link between Skp2 and p27, we asked whether there was a correlation between Skp2 and nuclear p27 levels in prostate cancer using the same set of tissue microarrays used for the Skp2 analysis. p27 expression (Fig. 1E) was well demonstrated in a subset of the 622 specimens (n = 175). As we observed in a previous study (18), nuclear p27 levels in histologically normal glandular epithelial cells adjacent to cancer were significantly higher when compared with that in cancer (Fig. 1E). Nuclear p27 staining in cancer cells was quantified for each cancer specimen and expressed as p27 labeling frequencies (%). They were then compared with the Skp2 labeling frequencies in the same set of specimens (Fig. 4A). A statistically significant inverse relationship between p27 and both Skp2 labeling frequencies and labeling index was evident (Spearman's rho = −0.310; $P = 0.0003$ for cell labeling frequency; Spearman's rho = −0.262; $P = 0.0021$ for cell LI).

Table 4 Univariate Cox proportional hazards models for recurrence-free survival time

| Prognostic marker	Hazard ratio	95% CI[a]	P
Skp2 LI (continuous)	1.004	1.000–1.007	0.0315
Skp2 LI (>10 *vs.* ≤10)	1.502	1.024–2.203	0.0376
Gleason score (prostatectomy)			
= 7 *vs.* ≤6	5.031	2.985–8.481	<0.0001
≥8 *vs.* = 7	2.263	1.413–3.625	0.0007
≥8 *vs.* ≤6	11.386	6.042–21.456	<0.0001
Seminal vesicle involvement	7.107	4.959–10.185	<0.0001
Positive surgical margins	3.856	2.647–5.618	<0.0001
ECE	6.116	3.913–9.559	<0.0001
Preoperative PSA	1.036	1.028–1.044	<0.0001
Positive lymph node	8.603	5.741–12.892	<0.0001
Clinical stage			
T_2 *vs.* T_1	3.063	1.822–5.151	<0.0001
T_3 *vs.* T_2	2.120	1.257–3.573	0.0048
T_3 *vs.* T_1	6.493	3.311–12.733	<0.0001

[a] CI, confidence interval.

In vitro studies linking loss of PTEN with increased Skp2 levels together with the genetic interactions seen between nuclear p27 loss and PTEN loss in prostate cancer (31, 32) led us to also compare expression of PTEN and Skp2 using the same set of prostate cancer tissue microarrays. A detailed analysis of PTEN expression in prostate cancer will be presented elsewhere.[4] In brief, PTEN expression varied in human prostate cancer, with some specimens exhibiting significant expression whereas other specimens exhibited low or undetectable expression (Fig. 1*F*). Interestingly, we found that increased Skp2 expression is significantly correlated with loss of PTEN staining (Fig. 4*B*; Spearman's rho = −0.14; *P* = 0.0444); tumors that induce Skp2 lack PTEN staining, whereas specimens maintaining PTEN expression tend to maintain low Skp2 levels.

Absence of Linkage of Cyclin A and Skp2 Expression in Prostate Cancer. Skp2 was originally identified based on its association with the S-phase cyclin, cyclin A, in complexes with Cdk2 (29). Cyclin A levels are known to be increased in prostate cancer (38), and we therefore sought to determine whether increased Skp2 and cyclin A levels were correlated. Cyclin A labeling frequencies were determined in 63 specimens and compared with Skp2 indices in the identical specimens. Although a positive correlation coefficient of 0.184 was found (Table 5), it was not statistically significant (*P* = 0.3314). Nevertheless, cyclin labeling frequencies alone significantly correlated with Gleason score, clinical stage, and ECE in this set of clinically confined cancer patients (Table 5). Moreover, cyclin A labeling was a predictive marker for recurrence-free survival time (*P* = 0.0041) as demonstrated in a Kaplan-Meier analysis in which the upper quartile of cyclin A labeling frequency (3.74%) was used as a cutoff level to differentiate between the patients (Fig. 5).

DISCUSSION

There is a critical need for improved assessment of the biological and clinical potential of prostate cancer to better
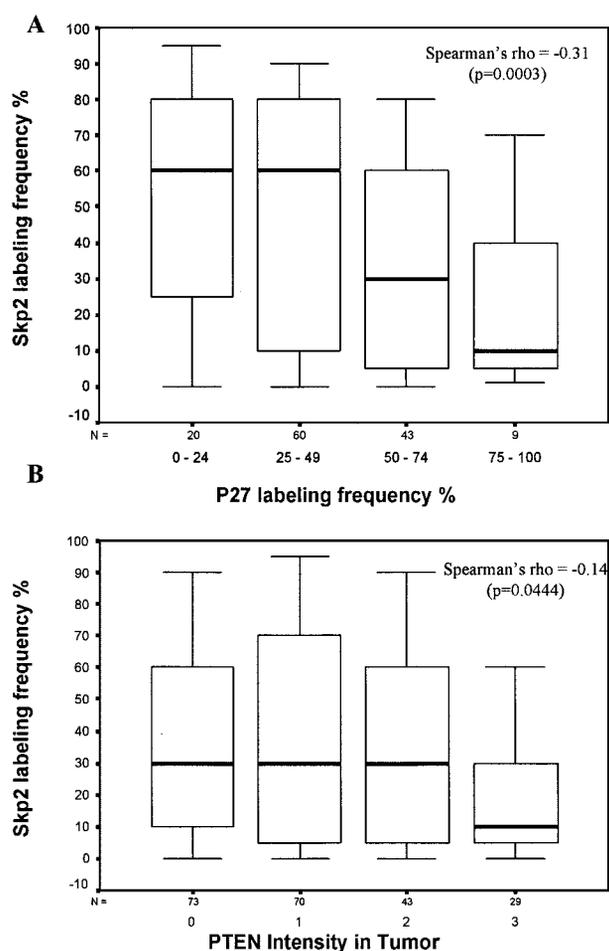
Fig. 4 Correlation of Skp2 with p27 and PTEN protein levels. Skp2 labeling frequency was negatively correlated with both p27 labeling frequency % and PTEN labeling intensity (*P* = 0.0003 and *P* = 0.0444, respectively; Spearman's correlation coefficient testing). Each box and the associated bars represent the values of middle 50% and the range of the data respectively. The dark line within a box denotes the median.

Table 5 Correlation of cyclin A with Skp2 and other clinical and pathological parameters

Cyclin A *versus*	N	rho[a]	P
Skp2 labeling frequency (%)	30	0.164	0.3865
Skp2 LI	30	0.184	0.3314
Gleason score[b]	63	0.268	0.0340
Positive surgical margins	63	0.048	0.7100
ECE	63	0.271	0.0314
Seminal vesicle involvement	63	0.097	0.4495
Clinical stage	63	0.293	0.0198
Preoperative PSA (ng/ml)	56	−0.102	0.4537

[a] Spearman correlation coefficient.
[b] Radical prostatectomy specimens.

stratify patients for treatment options. Prostate cancer is unique in the level of uncertainty regarding disease progression after initial diagnosis. Tumor specimens are often limited to biopsies, and although serum PSA has value in diagnosing prostate cancer, PSA values are limited in their capacity to accurately stage the disease or predict its course of progression (39). Therefore,
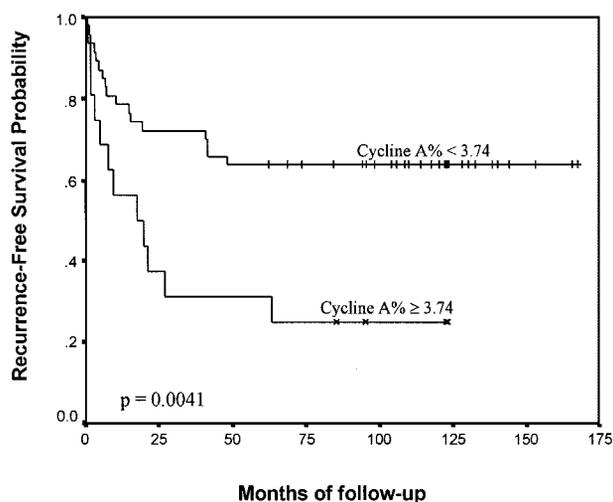
Fig. 5 Increased expression of cyclin A as a predictor of time to recurrence in prostate cancer patients. Comparisons between cancers with a cyclin A labeling frequency (%) <3.74 and those with a frequency ≥3.74 are shown.

continued investigations into the molecular mechanisms of prostate cancer development and progression offer hope toward establishing novel biomarkers for this disease and potentially generating therapeutic targets for improved treatment. In this study, we used an emerging technology, tissue microarrays, for high throughput assessment of specific biomarkers (Skp2 and p27) in prostate cancer specimens. These microarrays contain specimens from a large number (>600) of patients, thereby providing an opportunity to compare the expression patterns of multiple proteins in a single experiment over many cancer grades, which can be linked to clinical outcome via an associated database. This technology proved extremely efficient in generating useful expression data for Skp2 and p27 and provided a format to examine the relationship with PTEN.

Loss of p27 was previously linked with multiple cancer types, including prostate cancer. The recent identification of Skp2 as the specificity component of the E3 ubiquitin ligase responsible for p27 destruction led us to examine whether Skp2 might be linked to loss of p27 in prostate cancer. Three important findings were established:

(*a*) We found that Skp2 is overexpressed in premalignant lesions of the prostate (HGPIN) that are generally considered to be precursors of frank malignancies. To our knowledge, overexpression of Skp2 has not been demonstrated in premalignant lesions that have a predisposition toward loss of p27 expression. This temporal relationship implies a mechanistic relationship between increased Skp2 and decreased p27 that can be exploited clinically.

(*b*) We have found that Skp2 is overexpressed in prostate cancer tissues relative to normal prostate and has prognostic significance with respect to time to recurrence after radical prostatectomy. On the basis of Ki-67 and cyclin A staining, the frequency of Skp2 staining is not simply a result of proliferation. This information further defines Skp2 as being involved in prostate cancer development and progression and documents its prognostic capacities in a clinical setting. However, in multivariate analysis, the Skp2 LI did not provide an additional predictive value independent of other pathological markers for recurrence-free survival time (data not shown).

(*c*) We have found that Skp2 expression is inversely correlated with the expression of p27, as found previously in other cancer types (33–35), but is also inversely correlated with PTEN expression. The relationship between Skp2 expression and loss of PTEN is particularly interesting in light of the finding that deletion of PTEN in mouse fibroblasts leads to increased levels of Skp2 with concomitant reductions in p27 levels (32). Thus, it would appear that PTEN functions as a negative regulator of the Skp2 pathway that is normally used to control S-phase entry. Moreover, recent studies in the mouse have demonstrated that deletion of p27 in PTEN+/− mice leads to cooperation in transformation, with essentially 100% penetrance in the prostate (31). These results suggest that the presence of a single copy of PTEN is sufficient to suppress transformation and epithelial cell proliferation when p27 is present. These data together with our clinical analysis suggest that the decrease in p27 seen during prostate transformation involves accumulation of Skp2, possibly mediated by loss of PTEN expression. This model makes the testable hypothesis that transformation by PTEN would require an intact *Skp2* gene.

Alterations in the levels of proteins controlled by the ubiquitin-proteasome pathway during transformation are likely to be common, and further studies that define how such posttranscriptional control pathways are altered during transformation may provide additional biomarkers or facilitate the identification of novel therapeutic targets. Currently, there are ongoing clinical trials of small molecule drugs that target the proteasome, a general component of the ubiquitin-mediated proteolysis pathway (40). The finding that Skp2 is induced in a number of different cancers suggests that drugs directed at this molecule may provide a more selective target for therapeutic development.

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