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Evaluation of the Prognostic Potential of Hyaluronic Acid and Hyaluronidase (HYAL1) for Prostate Cancer[1]

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ABSTRACT

Despite the development of nomograms designed to evaluate the prognosis of a patient with prostate cancer (CaP), the information has been limited to prostate-specific antigen (PSA), clinical stage, Gleason score, and tumor volume estimates. To improve our ability to predict prognosis, information regarding the molecular properties of CaP is needed. Hyaluronic acid (HA) is a glycosaminoglycan that promotes tumor metastasis. Hyaluronidase (HAase) is an enzyme that degrades HA into angiogenic fragments. We recently showed that in CaP tissues, whereas HA is localized mostly in the tumor-associated stroma, HYAL1 type HAase is exclusively localized in CaP cells (Lokeshwar *et al.* J. Biol. Chem., *276:* 11922–11932, 2001). We evaluated the prognostic potential of HA and HYAL1 in CaP by immunohistochemistry.

Archival CaP specimens were obtained from patients who underwent radical retropubic prostatectomy for clinically localized CaP. Group 1 ($n = 25$) included patients who showed biochemical recurrence (PSA >0.4 ng/ml; mean recurrence: 21.3 months). Group 2 included patients with no clinical or biochemical recurrence ($n = 45$; mean follow-up: 80.9 months). For HA and HYAL1 staining, a biotinylated HA-binding protein and an anti-HYAL1 antibody were used. The staining was evaluated on the basis of intensity (0 to 3+) and as dense or sparse (for HA staining only) and then grouped as low grade and high grade.

In CaP specimens, HYAL1 was exclusively expressed in tumor cells. Although the stroma was stained positive for HA, 40% of tumor cells also expressed HA. HA, HYAL1, and combined HA-HYAL1 staining predicted progression with 96%, 84%, and 88% sensitivity, 55.5%, 80%, and 84.4% specificity, and 70%, 81.4%, and 85.7% accuracy, respectively. In the univariate analysis, preoperative PSA, Gleason sum, stage, margin, seminal vesicle, extra-prostatic extension (EPE), HA, HYAL1, and HA-HYAL1 were significant in predicting progression ($P < 0.05$). However, in the multiple logistic regression analysis, only EPE [odds ratio (OR) = 33.483; $P = 0.002$), HYAL1 (OR = 12.42; $P = 0.009$), HA-HYAL1 (OR = 18.048; $P = 0.0033$), and margin (OR = 26.948; $P = 0.006$)] were significant. Thus, in this 5-year follow-up study, HYAL1, together with EPE and margin, was found to be an independent prognostic indicator.

INTRODUCTION

In the last decade, because of the increased public awareness of serum PSA[3] screening, the number of CaP cases has steadily increased. PSA screening has resulted in the detection of more clinically localized CaP, which has the potential to be cured by radical prostatectomy or radiation therapy (1–4). However, within the first 10 years after surgery, CaP recurs [defined as PSA failure (biochemical relapse), local/systemic recurrence] in ~10–50% of cases, depending on a variety of prognostic factors (5–7). Treatment failure may be attributable to a local recurrence or distant metastasis. Existing preoperative indicators (*i.e.*, PSA levels, clinical stage, biopsy Gleason sum) or their combination in nomograms, as well as surgical and pathologic parameters (*i.e.*, prostatectomy Gleason sum, margin +/−, node status, seminal vesicle, and EPE), provide a limited estimate of the prognosis for CaP (8, 9). Identifying molecules that are expressed in clinically localized CaP but associate with CaP invasion and metastasis might significantly improve the prognostic capabilities and management of CaP patients after a curative approach. We have recently shown the expression of two tumor markers, HA and HAase, in CaP (10, 11).

HA is a glycosaminoglycan made up of repeated disaccharide units, D-glucuronic acid and *N*-acetyl-D-glucosamine (12–14). HA is a component of tissue matrix and tissue fluids. HA keeps the tissues hydrated and maintains the osmotic balance (12–14). In addition, by interacting through cell surface receptors (*e.g.*, CD44 and RHAMM), it regulates cell adhesion, migration, and proliferation (15). Concentrations of HA are elevated in several cancers, including colon, breast, prostate, bladder, and lung, and serve as highly sensitive and specific markers for detecting bladder cancer, regardless of the tumor grade (16–24). In tumor tissues, HA promotes tumor metastasis by opening up spaces for tumor cells to migrate and actively supports tumor cell migration by interacting with cell surface HA receptors (12, 14, 15). An HA coat around tumor cells may offer some protection against immune system surveillance and cause a partial loss of contact-mediated inhibition of cell growth and migration (25–28). Localization of HA either in tumor-associated stroma or tumor cells depends on the tissue origin. For example, in colon and stomach cancers, most of the tumor cells express HA (17, 21). In bladder cancer, HA expression is equally distributed in tumor-stroma and tumor cells (16). However, in CaP, HA is mostly localized in tumor stroma (22).

HAase is an endoglycosidase that degrades HA into small angiogenic fragments of 3–25 disaccharide units (29, 30). Angiogenic HA fragments induce endothelial cell proliferation, adhesion, and migration by activating focal adhesion kinase and mitogen-activated protein kinase pathways (31, 32). We have shown previously the presence of angiogenic HA fragments in high Gleason sum (≥7) CaP tissues (10). Six HAase genes have been identified in humans. Among these, products of HYAL1, HYAL2, and PH20 are well characterized (33–35). We have shown that HYAL1 type HAase is the major HAase expressed in prostate and bladder cancer tissues and have characterized the expression of HYAL1 at the mRNA and protein levels in prostate and bladder tumor cells (11, 36, 37). The HAase expressed by CaP cells has the same pH activity profile as that of HYAL1 (10). The expression of PH20 mRNA has been detected by reverse-transcription PCR analysis in some tumor tissues including CaP; however, the presence of PH20 protein has not been documented in these studies (38, 39). Because our recent observations show that the HAase family of genes are extensively alternatively spliced, which, in turn, regulates the generation of enzymatically active HAases, characterization of HAase expression in tumor tissues at the protein level may be nec-

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[3] The abbreviations used are: PSA, prostate-specific antigen; CaP, prostate cancer; EPE, extra-prostatic extension; HA, hyaluronic acid; HAase, hyaluronidase; IHC, immunohistochemistry; DAB, 3,3-diaminobenzidine; PPV, positive predictive value; NPV, negative predictive value; OR, odds ratio.

essary (40). By immunohistochemical techniques, we have shown previously that the HYAL1 type HAase is localized in tumor epithelial cells and the expression increases with higher grades of CaP (10). In this study, we examined the prognostic potential of HA and HYAL1 for predicting CaP progression by immunohistochemically localizing these markers in archival CaP tissues and correlating the staining intensity with PSA biochemical recurrence as an indicator of CaP progression.

MATERIALS AND METHODS

Specimens and Study Individuals. Seventy-three CaP specimens were obtained from patients who underwent radical retropubic prostatectomy for clinically localized CaP and were followed for recurrence/disease progression. Three of the total 73 specimens were not included in the final analysis because the patients from whom the those specimens were obtained had positive lymph nodes. On all of the patients, a minimum follow-up of 64 months was available. This study was conducted under a protocol approved by the Institutional Review Board of the University of Miami. The progressed group (group 1) of patients ($n = 25$) included those who had biochemical recurrence (PSA >0.4 ng/ml) within 64 months (mean time to recurrence: 21.3 months; range: 3 to 61 months). The nonprogressed group (group 2) included patients ($n = 45$) who were disease free (*i.e.*, no clinical or biochemical recurrence) for a minimum of 64 months (mean follow-up: 80.6 months; range: 64–114 months). The patient characteristics with respect to age, preoperative PSA, and tumor (*i.e.*, Gleason, stage, margin, EPE, seminal vesicle invasion) are shown in Table 1.

IHC. IHC localization of HA and HYAL1 in CaP tissues was carried out as described previously (10). For all specimens, paraffin-embedded blocks containing CaP tissues representing the majority of the Gleason sum were selected by the pathologist (F. C.) of the study. The blocks were cut into 3-μm thick sections and placed on positively charged slides. The specimens were deparaffinized, rehydrated, and treated with an antigen-retrieval solution (Dako Laboratories). For each specimen, two slides were prepared, one for HA and the other for HYAL1 staining. For HA staining, the slides were incubated with 2 μg/ml of a biotinylated bovine nasal cartilage protein at room temperature for 35 min (10). The specificity of HA staining was established as described previously (10). After incubation with the HA-binding protein, the slides were washed in PBS and sequentially incubated with streptavidin peroxidase at room temperature for 30 min and DAB chromogen substrate solution (Dako Laboratories). The slides were counterstained with hematoxylin, dehydrated, and mounted.

For HYAL1 staining, the slides were incubated with 3.7 μg/ml of anti-HYAL1 IgG at 4°C for 16 h. Rabbit polyclonal anti-HYAL1 IgG was generated against a peptide sequence present in the HYAL1 protein (amino acids 321 to 338), and its specificity for IHC was confirmed as described previously (10, 37). After incubation with anti-HYAL1 IgG, the slides were washed in PBS and incubated with a linking solution containing a biotinylated goat antirabbit IgG (Dako LSAB kit; Dako Laboratories) at room temperature for 30 min. The slides were then treated with streptavidin peroxidase and DAB chromogen. The slides were counterstained with hematoxylin, dehydrated, and mounted.

Slide Grading. Two readers (J. T. P. and V. B. L.) independently evaluated all slides in a blinded fashion. Any discrepancy in assigning staining intensity was resolved by both readers reexamining those slides simultaneously. The staining for HA and HYAL1 was graded as 0 (no staining), 1+, 2+, and 3+. For HA staining, both the tumor-associated stroma and tumor cells were graded separately in each slide for staining intensity. In the case of HA staining, the stromal staining was also evaluated as dense or sparse. The overall staining grade for each slide was assigned based on the staining intensity of the majority of the tumor tissue in the specimen. However, if ~50% of the tumor tissue stained as 1+ and the other 50% as 3+, the overall staining grade was 2+. If the staining distribution was ~50% of the tumor staining 2+ and the remaining staining as 3+, the overall staining inference was assigned as 3+. The staining scale was further subcategorized into low grade and high grade.

For HA staining, low-grade staining included 0, 1+ sparse, and 2+ sparse/dense staining, and high-grade staining included 3+ sparse and 3+ dense staining. In those cases ($n = 2$) where the stromal tissues were evaluated as low-grade staining but the tumor cells stained as 3+, the overall HA staining was considered as high grade. For HYAL1, high-grade staining represented 2+ and 3+ staining, whereas low-grade staining included 0 and 1+ staining intensities. For the combined HA-HYAL1 staining, a positive result was indicated only when both HA (stromal, tumor cells, or both) and HYAL1 staining intensities were of high grade. Any other combination was considered negative.

High-grade staining was considered as a true-positive result and the low-grade staining indicated a false-negative result if the patient had biochemical recurrence. If the patient had no clinical/biochemical recurrence, the low-grade staining indicated a true-negative result, and the high-grade staining was taken as a false-positive result.

Statistical Analysis. The consistency of staining was determined by staining 89% of the specimen for HA and HYAL1 staining twice on two separate occasions. The staining of these specimens was recorded independently. It is noteworthy that the interassay variability regarding staining intensity was determined by Pearson's correlation analysis. The Pearson's correlation coefficient (r) was 0.85 for HA staining and 0.9 for HYAL1 staining. Similarly, ~90% of the slides were recorded independently by each observer to determine consistency in grading. The sensitivity, specificity, accuracy, PPV, and NPV for HA, HYAL1, and HA-HYAL1 staining inferences were calculated using the 2×2 contingency table (high-grade/low-grade staining and progressed/nonprogressed CaP patients). The data on various, biochemical, surgical, and pathologic parameters, as well as HA, HYAL1, and HA-HYAL1 staining inferences, were analyzed by univariate logistic regression analysis. The data were also analyzed by a forward stepwise multiple logistic regression analysis beginning with all of the potential predictor variables. Statistical analysis was carried out by the statistician (R. C. D.) of the project using SAS Software Program (version 8.02; SAS Institute, Cary, NC).

Table 1 *Distribution of pre- and postoperative parameters among study patients*

Note that biochemical recurrence with postoperative PSA levels ≥0.4 ng/ml within 64 months was used as a cut point for defining progression. Thus, any CaP patient who showed a biochemical recurrence within 64 months was included in the progressed category. For the progressed group, median time for recurrence was 19 months and mean time for recurrence was 21.3 months. For the nonprogressed group, median follow-up time was 79 months; mean follow-up time was 80.6 months.

	Preoperative parameters			Postoperative parameters			
Progression	Age (yrs)	PSA (ng/ml)	Clinical stage	Gleason sum	EPE	Margin	Seminal vesicle invasion
Biochemical recurrence ($n = 25$)	Median: 64	Median: 9.0	T1 C = 10	6 = 2	(+) = 21	(+) = 18	(+) = 14
	Mean: 65.1	Mean: 14.04	T2 A = 5	7 = 14	(−) = 4	(−) = 7	(−) = 11
	Range: 51–74	Range: 2–62	T2 B = 10	8 = 6			
				9 = 3			
No biochemical or clinical recurrence ($n = 45$)	Median: 65	Median: 6.0	T_{1C} = 24	5 = 8	(+) = 6	(+) = 10	(+) = 3
	Mean: 63.5	Mean: 7.9	T_{2A} = 6	6 = 9	(−) = 39	(−) = 35	(−) = 42
	Range: 40–75	Range: 0.5–23	T_{2B} = 15	7 = 22			
				8 = 6			

RESULTS

HA Localization. HA was localized in 70 archival CaP specimens obtained from patients who underwent radical retropubic prostatectomy for clinically localized disease. These patients were being monitored for disease progression. An increase in PSA of ≥0.4 ng/ml was taken as an indicator of biochemical recurrence (either local recurrence or systemic progression). A minimum follow-up of 64 months was available for all patients. HA was localized in CaP tissues using a biotinylated HA binding protein. Shown in Fig. 1 are HA staining in Gleason sum 6 (*A* and *B*), 7 (*C* and *D*), and 8 (*E* and *F*) CaP specimens from nonprogressed (*A*, *C*, and *E*) and progressed (*B*, *D*, and *F*) patients. In all of the CaP specimens, HA was localized mostly in the tumor-associated stroma, although some tumor cells also showed HA staining in CaP specimens from patients who progressed (Fig. 1, *B* and *F*).

As shown in Fig. 1 *A*, *C*, and *E*, the CaP specimens from nonprogressed patients had low-grade HA staining, regardless of the Gleason sum. Of the 45 patients free of recurrence, 26 had low-grade HA staining. However, the CaP specimens from the patients who progressed in <64 months (median time to recurrence: 19 months; mean time to recurrence: 21.3 months) showed high-grade HA staining (Fig. 1, *B*, *D*, and *F*). Of the 25 CaP specimens from progressed patients, 22 showed high-grade staining in tumor stroma; 8 of these 22 also showed high-grade HA staining in tumor cells. In addition, 2 of the 25 CaP specimens showed high-grade staining only in tumor cells. Shown in Fig. 2 is an example of tumor cells that are positive for high-grade HA staining. Tumors cells appear to stain for HA equally, both in the cytoplasm and plasma membrane (Fig. 2*B*, *magnified view*). Thus, of the 25 CaP specimens from progressed patients, 24 showed high-grade HA staining either in tumor stroma or tumor cells or both. Thus, high-grade HA staining (stroma, tumor cells, or both) had 96% sensitivity, 55.5% specificity, 70% accuracy, 54.5% PPV, and 96.2% NPV for predicting the biochemical recurrence (Table 2).

HYAL1 Localization. An anti-HYAL1 peptide IgG was used to localize HYAL1 in the archival CaP specimens. Shown in Fig. 3 are HYAL1 localization in Gleason 6 (*A* and *B*), 7 (*C* and *D*), and 8 (*E* and *F*) CaP tissues from patients who did not (*A*, *C*, and *E*) and who did (*B*, *D*, and *F*) progress. As shown in Fig. 3, low-grade HYAL1 staining was seen in all three CaP specimens from patients who did not progress, regardless of their Gleason sum (Fig. 3, *A–E*). Among the 45 nonprogressed patients, CaP specimens of 36 patients showed low-grade HYAL1 staining. In CaP specimens from progressed patients, tumor cells, and not tumor-stroma, stained for HYAL1. All three CaP specimens from progressed patients showed high-grade HYAL1 staining (Fig. 3, *B*, *D*, and *F*), regardless of their Gleason sum. Of the 25 progressed patients, CaP specimens from 21 patients showed high-grade HYAL1 staining. Thus, in this cohort of 70 patients, the HYAL1 localization in CaP specimens had 84% sensitivity, 82.2% specificity, 82.9% accuracy, 70% PPV, and 90.2% NPV for predicting biochemical recurrence within 64 months (Table 2).

Combined HA-HYAL1 Staining. We next determined the ability of combined high-grade HA and HYAL1 staining to predict biochemical recurrence. The combined HA-HYAL1 staining has 84% sensitivity, 86.7% specificity, 85.7% accuracy, 77.8% PPV, and 90.7% NPV for predicting progression. When 64 months was used as a cutoff limit to evaluate progression, there were seven false-positive cases,

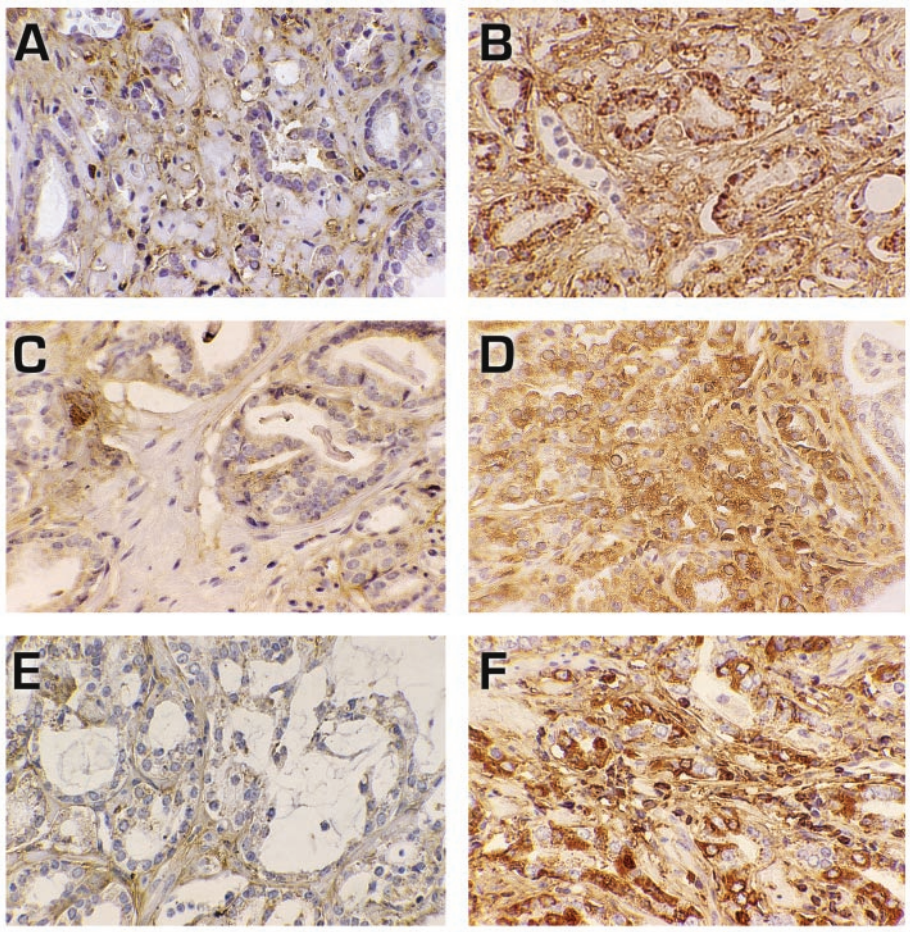

Fig. 1. HA staining of CaP specimens from nonprogressed and progressed patients. HA was localized in CaP tissues using a biotinylated HA-binding protein and a streptavidin peroxidase DAB-chromogen detection system. *A*, *C*, and *E*, HA staining in specimens from nonprogressed patients. *B*, *D*, and *F*, HA staining in specimens from progressed patients. *A* and *B*, Gleason 6 CaP; *C* and *D*: Gleason 7 CaP; *E* and *F*, Gleason 8 CaP. Each *panel* represents ×400 magnification.

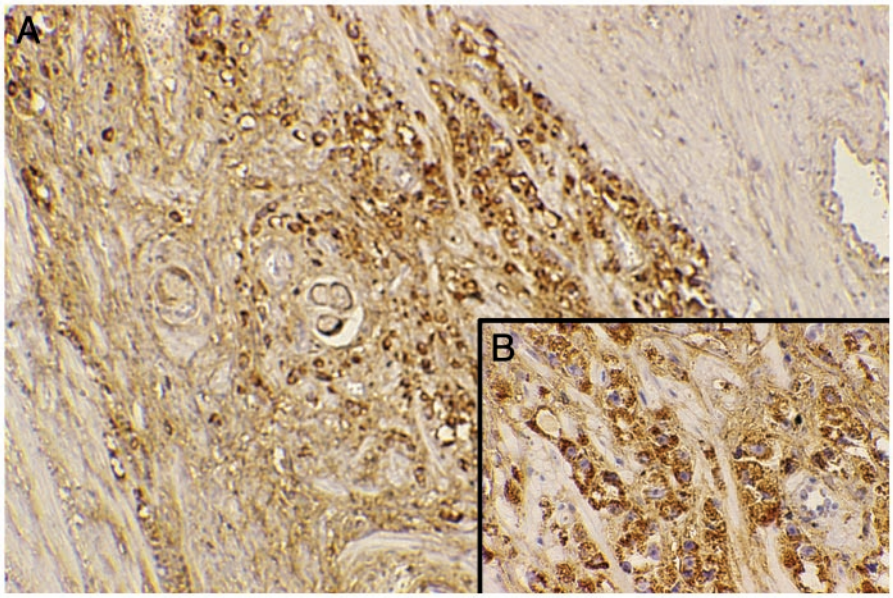

Fig. 2. A Gleason 8 CaP specimen with tumor cells showing HA staining. Gleason 8 specimen from a progressed patient where tumor cells show high-grade HA staining. *A*, ×100 magnification; *B*, ×400 magnification.

i.e., the specimens showed high-grade HA and HYAL1 staining, but the patients had no disease recurrence within 64 months. However, among these seven false-positive cases, two had a biochemical recurrence at 70 months (Table 2).

Evaluation of the Prognostic Capabilities of Preoperative, Postoperative Parameters, and HA and HYAL1 Staining Inferences: Univariate Analysis

We performed a univariate logistic regression analysis to determine the prognostic significance of preoperative parameters (*i.e.*, age, PSA, and clinical stage), postoperative surgical and pathologic parameters (*i.e.*, Gleason sum, margin +/−, EPE, seminal vesicle invasion +/−), as well as inferences of HA, HYAL1, and combined HA-HYAL1 staining. As shown in Table 3, both age ($P = 0.297$; OR = 1.041) and stage ($P = 0.287$; OR = 1.714) were not significant in predicting biochemical recurrence in the univariate analysis. However, preoperative PSA ($P = 0.0174$; OR = 1.10), Gleason sum ($P = 0.0023$; OR = 3.062), positive margin ($P = 0.0001$; OR = 9.0), EPE ($P < 0.0001$; OR = 34.125), seminal vesicle invasion ($P < 0.0001$; OR = 17.818), HA staining ($P = 0.0014$, OR = 30), HYAL1 staining ($P < 0.0001$; OR = 24.28), and combined HA-HYAL1 staining ($P < 0.0001$; OR = 34.125) were found to be significant in predicting biochemical recurrence. Patients with a Gleason sum ≥7 have been shown to have a greater risk of progression (41). In the univariate analysis, patients with a Gleason sum ≥7 had 2.3-fold greater odds of developing biochemical recurrence ($P = 0.015$; OR = 6.982) than when CaP tissues of all Gleason sums were analyzed together (Table 3).

Table 2 *Sensitivity, specificity, accuracy, PPV, and NPV of HA, HYAL1 and combined HA-HYAL1 staining inferences for predicting biochemical recurrence in CaP patients*

Note that 64 month follow-up was used a cut point for determining biochemical recurrence. Please note that 2 of the CaP patients who had a biochemical recurrence at 70 months and showed high-grade HA, HYAL1, and combined HA-HYAL1 staining were considered false positives and were included in the specificity calculation.

Parameters	HA	HYAL1	HA-HYAL1
Sensitivity	96% ($n = 24/25$)	84% ($n = 21/25$)	84% ($n = 21/25$)
Specificity	55.5% ($n = 25/45$)	82.2% ($n = 37/45$)	86.7% ($n = 39/45$)
Accuracy	70% ($n = 49/70$)	82.9% ($n = 58/70$)	85.7% ($n = 60/70$)
PPV	54.5% ($n = 24/44$)	70% ($n = 21/30$)	77.8% ($n = 21/27$)
NPV	96.2% ($n = 25/26$)	90.2% ($n = 37/41$)	90.7% ($n = 39/43$)

Evaluation of the Prognostic Capabilities of Preoperative, Postoperative Parameters, and HA and HYAL1 Staining Inferences: Forward Stepwise Multiple Logistic Regression Analysis. To determine the smallest number of variables that could jointly predict biochemical recurrence in this cohort of study patients, we performed the forward stepwise multiple logistic regression analysis. Initially, when age, preoperative PSA, clinical stage, Gleason sum, EPE, seminal vesicle invasion, HA staining, and HYAL1 staining were included in the model, only EPE ($P = 0.0023$; OR = 33.483), positive margin ($P = 0.0059$; OR = 26.948), and HYAL1 staining ($P = 0.0094$; OR = 12.423) reached statistical significance in predicting biochemical recurrence (Table 4*A*). Gleason sum did not reach statistical significance in the multiple logistic regression analysis even after the patients were stratified according to Gleason ≥7 and <7 (data not shown).

The inclusion of the combined HA-HYAL1 staining inference instead of the individual HA and HYAL1 staining inferences, in the multiple regression model, again showed that EPE ($P = 0.0023$; OR = 35.944), margin ($P = 0.0086$; OR = 24.438), and HA-HYAL1 staining ($P = 0.0033$; OR = 18.047) were significant in predicting biochemical recurrence (Table 4*B*). None of the other routine prognostic parameters [*i.e.*, age, PSA, clinical stage, Gleason sum (or Gleason stratification as Gleason ≥7 and <7 and seminal vesicle invasion) that were included in the model reached statistical significance (*i.e.*, $P > 0.05$ in each case)].

DISCUSSION

In this study, we demonstrate for the first time that HYAL1 and combined HA-HYAL1 staining inferences provide independent prognostic information for predicting CaP recurrence/progression over and above the prognostic information provided by the standard pre- and postoperative parameters. Radical prostatectomy for organ-confined disease is performed with the idea of disease cure (42, 43). However, disease relapses in a high percentage of cases despite careful selection of patients for surgery based on individual preoperative parameters or their combination in algorithms and nomograms (6). Certain genes and their products that associate with CaP growth and metastasis have shown a potential in predicting biochemical recurrence after surgery (8, 44). Because both HA and HYAL1 are likely to be involved in

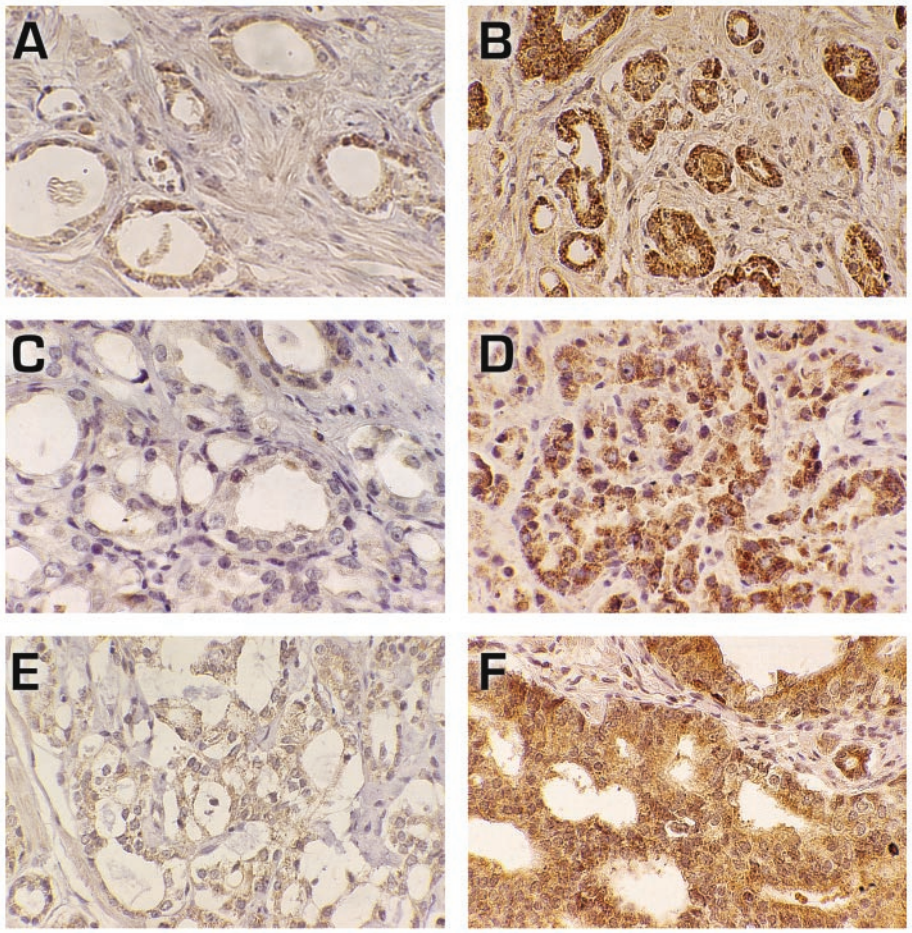

Fig. 3. HYAL1 staining of CaP specimens from nonprogressed and progressed patients. HYAL1 was localized in CaP tissues using an anti-HYAL1 antibody and a streptavidin peroxidase DAB-chromogen detection system. *A*, *C*, and *E*, HYAL1 staining in specimens from nonprogressed patients. *B*, *D*, and *F*, HYAL1 staining in specimens from progressed patients. *A* and *B*, Gleason 6 CaP; *C* and *D*, Gleason 7 CaP; *E* and *F*, Gleason 8 CaP. Each *panel* represents ×400 magnification.

tumor metastasis and angiogenesis (3, 45–49), it is not surprising that we found HA, HYAL1, and combined HA-HYAL1 staining inferences to have prognostic significance in predicting biochemical recurrence. In this study, we focused on CaP patients with clinically localized disease because such patients pose a dilemma for clinicians in terms of whether the cancer will metastasize; does the patient need additional treatment after surgery and what is the prognosis of the patient? Thus, in this patient population, accurate prognostic indicators will have clinical applicability. Nonetheless, although we eliminated from this study three CaP specimens from patients who had positive lymph nodes, all showed high-grade HA and HYAL1 staining, suggesting again that these molecules are associated with CaP progression.

In this study, HA staining in CaP tissues had high sensitivity (96%) but low specificity (55.5%) in predicting biochemical recurrence. Consequently, although in the univariate analysis, HA staining showed prognostic significance (Table 3), in the forward stepwise multiple logistic regression analysis, it had no additional prognostic significance when standard biochemical, surgical, and pathologic parameters, as well as HYAL1 staining, were included in the model (Table 4). Consistent with this observation, Lipponen *et al.* (22) reported previously that HA expression in tumor-stroma of CaP specimens has no additional prognostic significance over the standard prognostic indicators (*i.e.*, Gleason sum, stage, and node status). The combination of HA with HYAL1 did increase the specificity, accuracy, and PPV of the combination when compared with that of HYAL1 or HA alone (Table 2). Similarly, the OR of the HA-HYAL1 combination (18.047) was slightly better than that of HYAL1 (12.423) alone. Thus, based on our study and that by Lipponen *et al.* (22), HA staining of CaP tissues generally has low specificity and is probably not of clinical significance.

Lipponen *et al.* (22) also reported that 39% of the CaP specimens expressed HA in tumor cells in addition to the expression in tumor-stroma. However, the presence of HA in tumor cells did not have independent prognostic significance. In our study, although we found 40% of the CaP specimens from progressed patients showed high-grade HA staining in tumor cells, it did not have any independent prognostic significance. In contrast to these observations, in gastrointestinal, colon, and breast cancers, HA expression in tumor cells correlates with poor survival (17, 18, 21). Thus, although HA expression is elevated in many types of cancers, the clinical significance of

Table 3 *Univariate analysis of pre- and postoperative prognostic parameters and HA, HYAL1, and combined HA-HYAL1 staining inferences*

Parameter	χ^2	*P* value	OR	95% CI[a]
Age	1.088	0.297	1.041	0.965–1.122
PSA	5.652	0.0174[b]	1.1	1.017–1.192
Clinical stage	0.539	0.287	1.741	0.636–4.621
Gleason sum (Overall)	9.266	0.0023[b]	3.062	1.49–6.294
Gleason sum ≥7	5.919	0.015[b]	6.982	1.459–33.411
Margin	14.764	0.0001[b]	9.0	2.934–27.603
EPE	25.435	<0.0001[b]	34.125	8.655–134.545
Seminal vesicle invasion	15.969	<0.0001[b]	17.818	4.339–73.175
HA	10.222	0.0014[b]	30.0	3.729–241.337
HYAL1	22.627	<0.0001[b]	24.281	6.524–90.375
HA-HYAL1	25.435	<0.0001[b]	34.125	8.655–134.545

[a] CI, confidence interval.
[b] Statistically significant.

Table 4 *Forward stepwise multiple logistic regression analysis of pre- and postoperative prognostic parameters and HA, HYAL1, and combined HA-HYAL1 staining inferences*

The significant parameters ($P < 0.05$) selected by the model are shown. *A*, in the analysis, HA and HYAL1 staining inferences were included separately, along with other pre- (*i.e.*, age, PSA, and clinical stage) and post- [*i.e.*, Gleason sum (or stratified Gleason as ≥7 and <7), EPE, margin +/−, and seminal vesicle invasion] surgery parameters. *B*, combined HA-HYAL1 staining inference was included in the analysis, together with the above-mentioned pre- and postoperative parameters.

	A				B			
Parameter	χ^2	*P* value	OR	95% CI[a]	χ^2	*P* value	OR	95% CI
EPE	15.20	0.0023[b]	33.483	3.493–320.912	9.271	0.0023[b]	35.944	3.583–360.565
Margin	7.573	0.0059[b]	26.948	2.58–281.463	6.895	0.0086[b]	24.438	2.249–265.55
HYAL1	6.846	0.0094[b]	12.423	1.856–83.158	NA	NA	NA	NA
HA-HYAL1	NA	NA	NA	NA	8.628	0.0033[b]	18.047	2.619–124.378

[a] CI, confidence interval; NA, not applicable.
[b] Statistically significant.

this expression, in terms of predicting prognosis, varies with the tissue of origin of each cancer.

We have shown that HAase levels, measured using an ELISA-like assay, correlate with CaP progression and serve as accurate urine markers for detecting intermediate- and high-grade bladder cancer (11, 50). HYAL1 is expressed in invasive CaP and bladder cancer cells (11, 36, 37). The present study is the first to demonstrate the prognostic significance of HYAL1 expression in cancer. In this study, HYAL1 staining in CaP specimens had high sensitivity (84%), specificity (82.2%), and accuracy (82.9%) in predicting biochemical recurrence after radical prostatectomy. Among all of the pre- and postoperative prognostic parameters included in the forward stepwise multiple logistic regression analysis, only EPE and margin had additional prognostic significance if HYAL1 is included in the model (Table 4).

In contrast with our observation that HYAL1 expression correlates with biochemical in CaP, Hiltunen *et al.* (51) have shown that in malignant epithelial ovarian tumors, the concentration of HA and not HAase is elevated. These observations suggest that the clinical significance of HA and HYAL1 expression may be different in tumors of different tissue origins. This notion is corroborated by the observations that although HA expression correlates with malignant tumor progression in gastric, breast, and colorectal cancers (17, 18, 21), HA expression has no prognostic significance in CaP (results from this study and Ref. 22). In bladder cancer, the HYAL1 expression correlates with tumor grade, *i.e.*, high-grade tumors, which have a propensity to invade and metastasize express high levels of HYAL1 (16, 23). The differences in the clinical significance of HA and HAase expression in tumors of different origins is further supported by the observations that although HYAL1 expression in a transplantable rat colon carcinoma cell tumor suppresses tumor growth, the overexpression of HYAL1 induces metastasis in a human xenograft model involving PC3-M CaP cells (52, 53). Taken together, HYAL1 is a tumor cell-derived HAase, the expression of which associates with cancer progression.

In this study, all of the patients had a minimum follow-up of 64 months and a median follow-up of 79 months in the nonprogressed group. A follow-up duration of >5 years was long enough if any of the CaP patients placed in the nonprogressed category were to have a biochemical recurrence. Therefore, based on this first report, with sufficiently long follow-up, HYAL1 expression merits further investigation and HA expression is of limited clinical value in predicting biochemical recurrence after radical prostatectomy. Most CaP patients with clinically localized disease have preoperative PSA values between 4 and 10 ng/ml, stage T_{1C} disease, and a biopsy Gleason sum between 5 and 7 (8). Such similarity limits the prognostic capability of these preoperative parameters. Further studies on HYAL1 and HA-HYAL1 staining in biopsy specimens should reveal whether these markers could improve our ability to predict prognosis for patients who choose a curative approach involving radical prostatectomy.

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