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HYALURONIC ACID AND HYAL-1 EXPRESSION IN PROSTATE BIOPSY SPECIMENS: PREDICTORS OF BIOCHEMICAL RECURRENCE

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Abstract

Purpose—Molecular markers could aid PSA, biopsy Gleason sum and clinical stage in providing accurate information about prostate cancer (CaP) progression. HYAL-1 hyaluronidase and hyaluronic acid (HA) staining in prostatectomy specimens predicts biochemical recurrence. We examined whether HA and HYAL-1 staining in biopsy specimens predicts biochemical recurrence and correlates with the staining in matched prostatectomy specimens.

Materials and Methods—Biopsy and prostatectomy specimens were obtained from patients with clinically localized CaP (n = 61; mean follow-up = 103.1 months) from multiple centers; Gr. 1: patients with biochemical recurrence (n = 23); Gr. 2: patients without recurrence (n = 38). A biotinylated HA-binding protein and an anti-HYAL-1 antibody were used for HA and HYAL-1 staining. The staining was graded between 0 – 300 depending upon staining intensity and the area.

Results—HYAL-1 and HA were expressed in tumor cells and stroma, respectively. In biopsy specimens, HYAL-1 and HA expression was higher in Gr.1 (203.9 and 182.1) when compared to Gr. 2 (48.8 and 87.0; $P < 0.0001$). In univariate analysis, HA, HYAL-1, biopsy Gleason and PSA significantly predicted biochemical recurrence ($P < 0.001$). In multivariate analysis only HYAL-1 staining was an independent predictor of recurrence ($P < 0.001$; accuracy: 81.8%). In prostatectomy specimens only HYAL-1 staining correlated with the staining in biopsy specimens (Spearman $\rho = 0.72$; $P = 0.0002$), and predicted biochemical recurrence.

Conclusion—This is the first report that demonstrates that in biopsy specimens HYAL-1 staining is an independent predictor of biochemical recurrence and may be useful in selecting treatment.

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Keywords

Prostate cancer; Hyaluronic acid; Hyaluronidase; HYAL-1; Prognostic Markers; biopsy specimens

INTRODUCTION

Patients with clinically localized prostate cancer (CaP) are often treated with radical prostatectomy or radiation with curative intent. However, the disease recurs in a substantial number of the patients and becomes hormone refractory[1,2]. However, it is often difficult to identify those patients who will progress/recur and may need additional treatment. Currently available pre-operative parameters such as, biopsy Gleason sum, pre-operative prostate specific antigen (PSA), clinical stage are well investigated in terms of providing prognostic information biochemical recurrence. However, these parameters are often similar among patients who experience disease progression and those who do not. Molecular evaluation of biopsy specimens to identify tumors with invasive potential may allow individualization of treatment, including early adjuvant therapy for patients with aggressive CaP.

Hyaluronic acid is a glycosaminoglycan made up of repeating disaccharide units, D-glucuronic acid and N-acetyl-D-glucosamine. HA regulates several cellular functions, including adhesion, migration and proliferation. HA concentration is elevated in a variety of tumors[3,4]. HA promotes tumor growth and metastasis, however, the prognostic potential of HA appears to depend upon the tissue origin of the tumor[3–6]. For example, we have shown that the HA test (i.e., measurement of urinary HA levels) is an accurate marker for diagnosing bladder cancer, regardless of tumor grade[7]. However, HA is not an independent prognostic marker for predicting muscle invasion by a bladder tumor and for predicting biochemical recurrence following radical prostatectomy[8–10]. Contrarily, high level HA expression in tumor cells correlates with poor prognosis in terms of disease progression, and shortened overall and disease-specific survival in the carcinomas of the gastrointestinal tract, breast and ovary (reviewed in ref [3]). There is some evidence that HA may promote progression of CaP to become androgen independent[11]. We have previously shown that in radical prostatectomy specimens, HA expression is elevated in CaP tissues, but it is not an independent predictor of biochemical recurrence [9,10].

Hyaluronidase (HAase) is an endoglycosidase that degrades the HA polymer. HYAL-1 is the major tumor-derived HAase and its expression in tumor cells correlates with aggressiveness of the tumor[3,12]. Elevated urinary HYAL-1 level (i.e., measured as the HAase test) is an accurate marker for detecting high-grade bladder cancer. Therefore, when combined with the HA test, the HA-HAase test has about 90% sensitivity and ~ 85% accuracy in detecting bladder cancer[7,13]. HYAL-1 is also an independent prognostic predictor of muscle invasive bladder cancer[8]. We have previously shown that silencing HYAL-1 expression in bladder and CaP cells inhibits tumor growth, lymph node, vascular and lymphatic invasion and angiogenesis[14,15]. We have shown that HYAL-1 expression in radical prostatectomy specimens is an independent prognostic indicator for predicting biochemical recurrence[8,9].

In this study, we evaluated HA and HYAL-1 expression in prostate biopsy specimens and correlated in with biochemical recurrence. Tissue fixation and preservation differs in various institutions, academic or community hospitals, which can affect a marker's performance. Therefore, in this study, the biopsy specimens were obtained from different hospitals. Tumor heterogeneity is inherent in CaP specimens, and therefore, we also determined HA and HYAL-1 expression in matched radical prostatectomy specimens.

MATERIALS AND METHODS

Specimens and patients

We analyzed 61 matched biopsy and radical prostatectomy specimens from CaP patients who underwent radical prostatectomy with bilateral lymphadenectomy between 1992 and 2001. This study was approved by University of Miami's Institutional Review Board. During the nine year period, 911 patients underwent radical prostatectomy, of which 695 did not receive neoadjuvant androgen deprivation therapy. Out of these 695 patients, well preserved tissue blocks for preparing unstained slides could be retrieved for 250 patients. A single surgeon (MSS) had performed radical prostatectomy on all of these patients at University of Miami. Since these 250 patients had undergone prostate biopsy at various hospitals (n = 11) in South Florida, we requested unstained tumor positive biopsy specimen slides from these hospitals. We were able to obtain 69 specimens, out of which 8 could not be evaluated either due to the loss of tissue during staining procedure. Of the 61 patients with stainable biopsy specimens, 23 patients experienced biochemical recurrence following radical prostatectomy and 38 were free of recurrence. The median follow-up on all patients was 99 months (mean follow-up 103.1 months, 95% confidence interval (CI) 85.6 – 109.7 months). The median time to recur was 36 months (mean 40.6 months, 95% CI 11 – 65 months).

Biochemical recurrence was defined as a PSA ≥ 0.4 ng/dL in 2 successive measurements following radical prostatectomy; the date of the first measurement was taken as the date of recurrence. Pre-operative patient characteristics are shown in Table 1.

Immunohistochemistry

Each biopsy and radical prostatectomy specimen was evaluated to ascertain the presence of tumor in the specimen and the Gleason sum. Biopsy and radical prostatectomy specimen slides were deparaffinized, rehydrated and treated with an antigen retrieval solution (Dako, Glostrup, Denmark). HA was localized in these specimens using a biotinylated HA-binding protein (1 μg/ml; room temperature for 2 hours), purified from bovine nasal cartilage[16]. HYAL-1 was localized using a rabbit polyclonal antiHYAL-1 IgG (1 μg/ml; 37°C). We have previously described the nature and specificity of the HA-binding protein and antiHYAL-1 IgG[9,17]. Following incubation with primary reagents, the slides were incubated with LASB solution (Dako) and 3, 3′-diamonobenzidine staining. The sides were counterstained with hematoxylin. To determine the reproducibility of staining, all biopsy slides and 25% of prostatectomy slides were stained twice and scored independently.

Slide grading

Each slide was graded by three readers (CG, PG and VBL) independently, and in a blinded fashion. In each slide, the tumor areas stained for HA and HYAL-1 were graded for staining intensity (0 – 3+) and then multiplied by the area of staining. Thus, each specimen could receive a score between 0 and 300 for HYAL-1 and HA staining. The scores of the three readers were average to get the final score. Overall there was 90% agreement between the scores of the readers. Any discrepancy was resolved by two readers reading the slide simultaneously. The slides were also evaluated using an IP image analysis software and there was significant correlation between the average scores of the readers and IP image analysis scores (Spearman $\rho = 0.851$; 95% CI: 0.723 ± 0.927; $P < 0.001$).

Statistical analyses

To determine the correlation of each clinical/pathologic parameter and HA and HYAL-1 staining scores (analyzed as continuous variables) with biochemical recurrence, we performed logistic regression single parameter analysis. Since this is a retrospective cohort

study, we performed the Cox proportional hazard analysis, by including all clinical/pathologic parameters and HA and HYAL-1 staining scores (biopsy or radical prostatectomy) in the model, to determine which parameters jointly predict biochemical recurrence. Kaplan-Meier plots were generated to determine whether HA and HYAL-1 could stratify the patient cohort as recurred versus non-recurred. Correlation between biopsy and radical prostatectomy specimens with respect to HA and HYAL-1 staining scores was evaluated by Pearson's correlation analysis. Receiver operating characteristic curves were generated to determine the association between staining scores and biochemical recurrence. The cut-off values that yielded highest sensitivity (1-specificity) values, were used to define high and low expression of HA (cut-off: 120) and HYAL-1 (cut-off: 185). These cut-off values were calculated using the JMP 6 software (SAS Institute, Carey NC USA). The sensitivity and specificity for HA and HYAL-1 staining inferences were calculated and cross-validated to obtain mean ± SD and 95% CI, as described before[18].

RESULTS

Correlation of HA and HYAL-1 staining in biopsy specimens with biochemical recurrence

We stained 61 biopsy specimens to evaluate HA and HYAL-1 expression. Figure 1, shows HA and HYAL-1 staining in biopsy specimens from patients with Gleason 7 CaP. HA staining is high in the specimen from a patient who experienced biochemical recurrence following radical prostatectomy, when compared to the patient who did not experience biochemical recurrence (Figure 1A). HA staining is localized primarily in tumor-associated stroma. While there is minimal HYAL-1 expression in prostate tumor in the biopsy specimen from patient who did not experience biochemical recurrence, the HYAL-1 staining is high in the specimen from a patient who had biochemical recurrence (Figure 1A). As we have reported previously, HYAL-1 expression is exclusively in tumor cells[9,10] The differences in the mean ± SD intensity scores for both HA ($P = 0.0003$) and HYAL-1 ($P < 0.0001$), among patients who experienced biochemical recurrence and those who did not, are statistically significant (Kruscal-Wallis test; Figure 1B).

To determine whether age, pre-operative PSA, biopsy Gleason sum, clinical stage and/or the staining scores HA/HYAL-1 predict biochemical recurrence, we performed logistic regression analysis (univariate analysis). As shown in Table 2, pre-operative PSA, Gleason sum (overall and stratified), and HA and HYAL-1 staining intensity scores correlate with biochemical recurrence.

HA and HYAL-1 staining intensity scores in biopsy specimens independently correlate with biochemical recurrence

To determine which of the clinical and pathologic parameters and/or HA and HYAL-1 staining inferences predict biochemical recurrence, we performed the Cox proportional hazards analysis. When only clinical and pathologic parameters (i.e., age, biopsy Gleason, pre-operative PSA and clinical stage) were included in the model, biopsy Gleason sum reached statistical significance (χ^2: 5.21; $P = 0.025$ Risk ratio 1.48 95% CI: 1.06 – 2.06). As shown in Table 2, when HA staining score is included in the model, along with all clinical and pathologic parameters described above, HA staining is the only parameter that reached statistical significance in predicting biochemical recurrence. However, when biopsy Gleason sum is categorized as ≥ 7 and < 7, both HA and Gleason sum reach statistical significance (data not shown). When HYAL-1 is included in the model, only HYAL-1 staining reaches statistical significance, regardless of whether Gleason sum is included as a continuous or a categorized variable. When both HA and HYAL-1 are included in the model, again, only HYAL-1 reaches statistical significance (Table 3). These results show that HYAL-1 staining

in biopsy specimens (and HA staining if HYLA-1 staining scores are absent) is potentially an independent predictor of biochemical recurrence.

We next determined whether HA and HYAL-1 staining inferences stratify patients as those who recurred and those who did not. The Kaplan-Meier plot in Figure 1C shows that patients with high HA staining (≥ 120) recurred faster (50% within 73 months; log rank test: χ^2: 12.75; p value = 0.0004) than with low HA staining (20% after 78 months). Similarly, patients with high HYAL-1 staining (≥ 185) recurred faster (50% within 49 months; log rank test: χ^2: 34.56; p value < 0.0001) than with low HYAL-1 staining (10% after 80 months).

We next evaluated whether HA, and more importantly, HYAL-1 can accurately predict biochemical recurrence. As shown in Table 4, HA staining has reasonable sensitivity but low specificity to predict biochemical recurrence, whereas, HYAL-1 staining has both high sensitivity and specificity to predict biochemical recurrence. Although, the sample size in this study is relatively small, and the sensitivity and specificity of HA or HYAL-1 staining to predict biochemical recurrence will require independent confirmation, the cross-validation results presented in Table 4, strengthen the results.

Correlation between HA and HYAL-1 expression, in biopsy and radical prostatectomy specimens

To determine whether the HA and HYAL-1 expression in CaP detected in biopsy specimens was representative of the expression in the final pathology specimens, we stained the radical prostatectomy specimens from the same patients for HA and HYAL-1. Figure 1D, shows HA and HYAL-1 staining in radical prostatectomy specimens from patients with Gleason sum 7 CaP. In these specimens, HA and HYAL-1 staining is high if the patient had biochemical recurrence when compared to the specimens from patients who did not recur. Pearson's correlation analysis (non-parametric) showed significant correlation between HYAL-1 expression in biopsy and radical prostatectomy specimens (Spearman $\rho = 0.719$; 95% CI: 0.4052 to 0.8813; p = 0.0002). However, the correlation between HA expression in biopsy and radical prostatectomy specimens was not statistically significant (Spearman $\rho = 0.275$; 95% CI: −0.167 to 0.625; p = 0.205). In the multivariate analysis that included clinical (age, pre-operative PSA, clinical stage) and pathologic parameters (Gleason sum, EPE, seminal vesicle invasion and margin) and HA and HYAL-1 staining scores, only HYAL-1 staining (χ^2: 9.58; P = 0.002 Risk ratio 1.01 95% CI: 1.00 – 1.03). The sensitivity and specificity data presented in Table 4 show that, using the same cut-off value (i.e., 120) as for the biopsy specimens, HA staining in radical prostatectomy specimens has high sensitivity but poor specificity to predict biochemical recurrence. Contrarily, as in the case of biopsy specimens, HYAL-1 staining in radical prostatectomy specimens predicted biochemical recurrence with high sensitivity and reasonable specificity.

DISCUSSION

Although the widespread acceptance of PSA has increased the number of CaP cases detected and cured each year, the majority of the newly detected patients have biopsy Gleason between 6 and 7 and serum PSA between 4 and 10-ng/ml. Thus, individualized treatment may be possible if molecular markers that function in promoting invasion/metastasis are added as adjuncts to the current diagnostic tools[19]. Consistent with this thesis, in this study we found that HA, but more importantly HYAL-1 expression in biopsy specimens independently predicts biochemical recurrence following radical prostatectomy.

HA due to its ability to promote cell migration and motility supports tumor metastasis, but it is HYAL-1 that often correlates with the aggressiveness of the tumor[4]. For example,

HYAL-1 is necessary for cell cycle progression and invasive activity of CaP cells *in vitro* and to promote tumor growth and vascular/lymphatic invasion *in vivo*[14]. Moreover, it has been consistently shown that in addition to HA, HYAL-1 expression is necessary for CaP metastasis[6,20]. Consistent with the dependence of HA on HYAL-1 to promote metastasis, in this study we found that when HYAL-1 staining inference is added to the multivariate model, it erases the independent prognostic significance of HA expression in biopsy specimens. Furthermore, in this study, as well as, in two previous studies we found that HA expression in radical prostatectomy specimens is not an independent prognostic predictor of biochemical recurrence and two independent studies have confirmed these findings[20,21].

Although the prognostic significance of HA to predict biochemical recurrence is limited, HYAL-1 expression in both biopsy and radical prostatectomy specimens appears to be a strong predictor of biochemical recurrence (this study and ref. [8,9]). The discrepancy in these two functionally interdependent markers, with respect to prognostic significance, may be because HA expression is mainly stromal, whereas, HYAL-1 is expressed exclusively by invasive tumor cells. In fact, silencing HYAL-1 expression in prostate xenografts decreases HA expression in the tumor-associated stroma[11]. Thus, HYAL-1 may be a molecular switch that controls the metastatic promotion of CaP by the HA-HYAL-1 system.

Tumor heterogeneity in CaP is a significant factor that impedes the accurate prognostic prediction of tumor behavior based on clinical and pathologic parameters, as well as molecular markers available at the biopsy stage. For example, case of p53 and bcl-2, although the staining in biopsy specimen correlates with biochemical recurrence, the staining in the biopsy specimens for both markers does not correlate with the staining in radical prostatectomy specimens.[23] In our study there was upstaging in Gleason sum in 44% of the specimens (27 out of 61). However, it is noteworthy that HYAL-1 staining scores in biopsy specimens significantly correlated with the scores in radical prostatectomy specimens, suggesting that molecular markers that correlate with invasive behavior of the tumor may help in making better prognostic predictions at the biopsy stage.

This is the first study that has evaluated HYAL-1 and HA expression in prostate biopsy specimens. The clinical significance of our study is that current preoperative parameters do not provide adequate information regarding which patients will remain disease free versus those who will experience disease progression despite radical prostatectomy. Our study shows that HYAL-1 staining very likely has the potential to not only predict biochemical recurrence at the radical prostatectomy stage, but also at the biopsy stage. Such information at the biopsy stage may help the physicians to stratify patients into different risk categories for biochemical recurrence and identify those patients who will need additional treatments following radical prostatectomy. The biopsy specimens were obtained from multiple community centers and the same markers were evaluated in the matched biopsy and radical prostatectomy specimens. However, this was a retrospective cohort study and the sample size was limited. Taking into consideration some of these caveats, a prospective multicenter center will need to be conducted to fully assess the prognostic potential of HYAL-1.

Conclusions

HYAL-1 staining in biopsy specimen has potential to aid existing clinical and pathologic parameters to stratify patients developing biochemical recurrence following radical prostatectomy. If these results can be confirmed in prospective multicenter study, it could help in individualizing treatment options.

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Abbreviations used

CaP	Prostate cancer
HA	Hyaluronic acid
HAase	Hyaluronidase
PSA	prostate specific antigen

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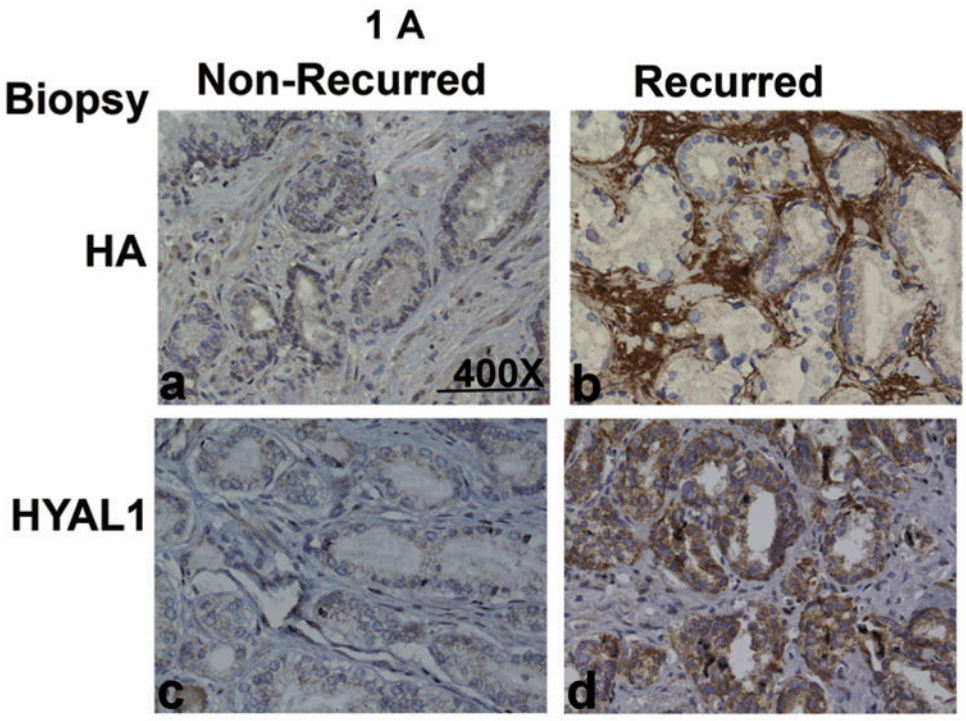


Figrue 1 B

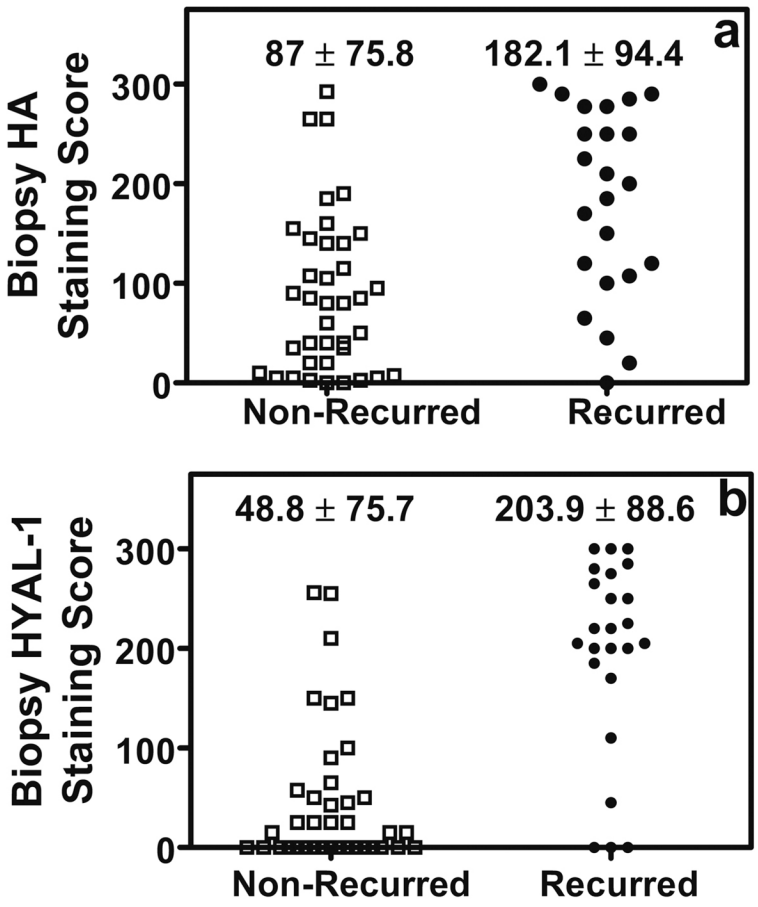
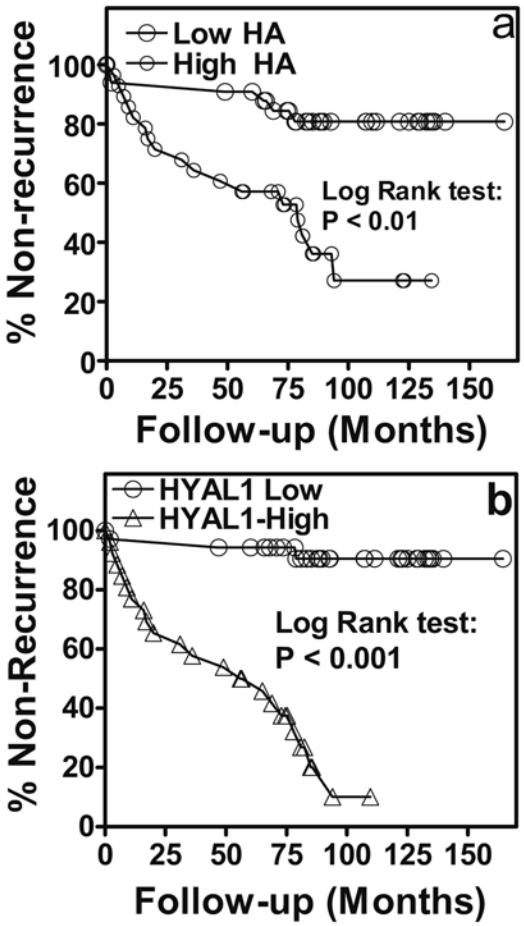


Figure 1 C



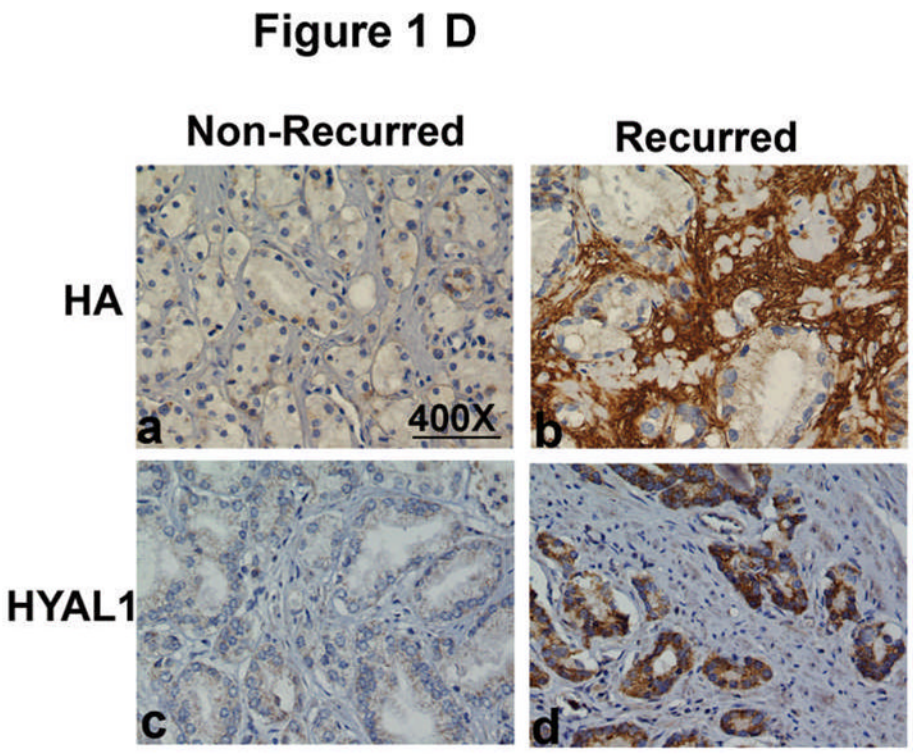


Figure 1. Localization of HA and HYAL-1 in CaP specimens

A. HA and HYAL-1 expression in biopsy specimens from patients with Gleason 7 CaP who either had biochemical recurrence or did not recur. a, b: HA staining; c, d: HYAL-1 staining. a, c: non-recurred; b, c: recurred. Magnification: 400X. **B:** Scatter diagram of HA and HYAL-1 staining scores in CaP specimens, from patients who either had biochemical recurrence or did not recur. The mean ±SD scores for HA (a) and HYAL-1 (b) staining intensity are indicated. Each specimen could receive a score between 0 and 300. **C:** Kaplan-Meier plots were created to stratify patients with respect to biochemical recurrence based on HA (a) or HYAL-1 (b) staining intensity scores in biopsy specimens. **D:** HA and HYAL-1 expression in radical prostatectomy specimens from patients with Gleason 7 CaP who either had biochemical recurrence or did not recur. a, b: HA staining; c,d : HYAL-1 staining. a, c: non-recurred; b, c: recurred. Magnification: 400X.

Table 1

Patient characteristics

The information on the pre- and post-biopsy parameters was assessed by pathologic examination of the surgical specimens (Gleason sum (biopsy or radical prostatectomy, margin status, extra prostatic extension (EPE), seminal vesicle invasion and lymph node status) and the available clinical information (age, PSA and clinical stage). In this study all patients were node negative. Therefore, these parameters are considered as baseline covariates. Mean ± SD staining scores for HA and HYAL1 are shown.

Characteristics	Recurred	Non-Recurred
Number of patients	23	38
Age	Mean: 62.4 Median: 65; 95% CI: 57 – 66	Mean: 61.1 Median: 63; 95% CI: 59 – 65
Parameters available at biopsy stage		
PSA	Mean: 12.8 Median: 13; 95% CI: 6.7 – 15.7	Mean: 8.3 Median: 7.3; 95% CI: 6 – 8.4
Gleason sum	GL6=10 GL7=5 GL8=4 GL9=4	GL6=28 GL7=9 GL9=1
Clinical Stage[a]	T1C=14 T2A=5 T2B=3 T3=1	T1C=20 T2A=6 T2B=10 T3=2
HA staining	182.1 ± 94.1	87 ± 75.8
HYAL1 staining	203.9 ± 88.6	48.8 ± 75.7
Parameters available after prostatectomy		
Gleason sum	GL5= 0 GL6= 4 GL7= 8 GL8= 6 GL9= 5	GL5=2 GL6=14 GL7=15 GL8=6 GL9=1
EPE	(+) 14 (−) 9	(+) 4 (−) 34
Pathologic Stage	T2 = 6 T3a = 7 T3b = 10	T2 = 34 T3a = 1 T3b = 3
Seminal vesicle invasion	(+) 10 (−) 13	(+) 3 (−) 35

Characteristics	Recurred	Non-Recurred
Margin	+) 14 (−) 9	(+) 8 (−) 30
HA staining	236 ± 81.1	125 ± 96.4
HYAL1 staining	266.3 ±53	86.7 ± 89.5

Table 2

Univariate Analysis of pre- and post-biopsy parameters and HA and HYAL-1 staining inferences

Logistic regression single parameter analysis was used to determine the association of clinical (age, pre-operative PSA, and clinical stage) parameters and biopsy Gleason sum and HA and HYAL-1 staining inferences with biochemical recurrence.

Parameter	Chi-Square	P-value	Odds Ratio	95% CI
Age	0.89	0.33	0.97	0.89 – 1.06
[a] Gleason sum (GS)	7.49	0.0062[b]	2.02	1.28 – 3.55
GS ≥ 7	6.36	0.012[b]	2.05	1.19 – 3.64
GS ≥ 8	7.3	0.007[b]	4.44	1.8 – 19.6
Clinical Stage	0.114	0.736	1.11	0.604 – 2.11
HA	14.86	0.001[b]	1.01[c]	1.00 – 1.03
HYAL-1	31.99	0.0001[b]	1.02[c]	1.01 – 1.03
PSA	7.6	0.006[b]	1.12[c]	1.03 – 1.23

[a] Gleason sum was either continuous or categorized.

[b] Statistically significant;

[c] Change in odds ratio per unit change in the parameter.

Table 3

Multivariate analyses of pre- and post-biopsy parameters and HA and HYAL-1 inferences

Cox proportional hazard analysis was performed by including either HA (a), HYAL-1 (b) or HA and HYAL-1 (c: as individual continuous variables) staining inferences together with clinical parameters (i.e., age, PSA, and clinical stage) and biopsy Gleason sum.

Parameter	Chi-Square	P-value	Risk Ratio	95% CI
HA[a]				
HA	6.93	0.009	1.00*	1.00 – 1.01
HYAL-1[b]				
HYAL-1	26.93	< 0.001	1.01*	1.00 – 1.02
HA and HYAL-1[c]				
HYAL-1	20.11	< 0.001	1.01*	1.00 – 1.02

* Change in risk ratio per unit change in HA or HYAL-1 staining score.

Table 4

Determination of sensitivity and specificity of HA and HYAL-1 staining inferences for predicting biochemical recurrence

The sensitivity, specificity and accuracy for HA and HYAL-1 staining inferences in biopsy and radical prostatectomy specimens were calculated using the cut-off limits determined from the ROC curves and then cross-validated. The data presented in the parentheses are mean values and 95% CI, derived from the cross validation analyses.

Parameter	Area Under the Curve	Sensitivity	Specificity	Accuracy
HA (Biopsy)	0.77	72.7% (71.5%; 95% CI: 60.5 – 76.5)	63.7% (63.9%; 95% CI: 60.4 – 72.2)	68.2% (67.7%; 95% CI: 62.9 – 75.3)
HA (prostatectomy)	0.79	90.9% (85.8%; 95% CI: 80.6 – 93.5)	55.2% (56.3%; 95% CI: 51.7 – 63.8)	73% (71.8%; 95% CI: 67.6 – 77.4)
HYAL-1 (Biopsy)	0.87	81.8% (82.3%; 95% CI: 76.6 – 92.3)	81.8% (81.9%; 95% CI: 76.3 – 86.8)	81.8% (81.4%; 95% CI: 76.9 – 88.4)
HYAL-1 (prostatectomy)	0.95	90.9% (88.9%; 95% CI: (81.3 – 95.6)	75% (76.6%: 95% CI: 72.3 – 81.4)	83% (82.6%; 95% CI: 77.1 – 87.5)