AD_____

Award Number: DAMD17-02-1-0095

TITLE: Hyaluronic Acid and Hyaluronidase in Prostate Cancer: Evaluation of Their Therapeutic and Prognostic Potential

PRINCIPAL INVESTIGATOR: Vinata B. Lokeshwar, Ph.D.

CONTRACTING ORGANIZATION: University of Miami School of Medicine
Miami, Florida 33136

REPORT DATE: January 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	January 2004	Annual (1 Jan 2003 - 31 Dec 2003)

4. TITLE AND SUBTITLE
Hyaluronic Acid and Hyaluronidase in Prostate Cancer: Evaluation of Their Therapeutic and Prognostic Potential

5. FUNDING NUMBERS
DAMD17-02-1-0095

6. AUTHOR(S)
Vinata B. Lokeshwar, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Miami School of Medicine
Miami, Florida 33136

E-Mail: vlokeshw@med.miami.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)
The limited knowledge about which clinically localized prostate cancer (CaP) would progress, and when it will recur severely impedes individualization of therapy and prediction of outcome. Several molecules that function in cancer growth and progression can serve prognostic indicators. Treatment modalities that target the functions of these molecules could effectively control CaP progression. We have studied two such molecules hyaluronic acid (HA) and HYAL1 type hyaluronidase (HAase). HA is a glycosaminoglycan and HAase is an enzyme that degrades HA into angiogenic fragments. Immunohistochemical analysis using archival radical prostatectomy CaP specimens from patients on whom theree is minimum 6-year follow-up (range: 72 - 131 months) show that HYAL1 and combined HA-HYAL1 staining are independent predictors for biochemical recurrence. Functional analysis of HYAL1 by stable cDNA transfection and in vitro and in vivo studies show that both over and under production of HYAL1 severely inhibits CaP growth and its invasive potential. Use of 3 HAase inhibitors show that inhibition of HAase activity also inhibits CaP cell proliferation. The degree of inhibition show that inhibition of HAase activity also inhibits CaP cell proliferation. The degree of inhibition of cell proliferation is dependent on the degree of inhibition of HAase activity. Ongoing studies are examining the effect of HAase inhibition on gene expression by cDNA microarray analysis.

14. SUBJECT TERMS
No Subject Terms Provided.

15. NUMBER OF PAGES
48

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Cover.....

SF 298.....

Table of Contents.....

Introduction.....1

Body.....2-12

Key Research Accomplishments.....12

Reportable Outcomes.....12-13

Conclusions.....13

References.....13-16

Appendices.....17-45

1. Posey, TJ, Soloway, MS, Ekici, S, Sofer, M, Civantos, F, Duncan, RC, Lokeshwar, VB,. Cancer Research 63:2638-2644, 2003

2. Ekici, S, Cerwinka, W H, Duncan, RC, Gomez, P, Civantos, F, Soloway, MS, Lokeshwar, VB. MS submitted to Journal of Cancer

Principal Investigator: Vinata B. Lokeshwar, Ph.D.

Project Title: Hyaluronic acid and hyaluronidase in prostate cancer: Evaluation of their therapeutic and prognostic potential.

1. A. INTRODUCTION AND OBJECTIVES: There are two overall objectives of this funded project. The first is to evaluate the independent prognostic potential of two tumor markers, identified in our laboratory, i.e., hyaluronic acid (HA) and HYAL1 type hyaluronidase (HAase). The second objective is to evaluate the effect of HYAL1 inhibition on prostate cancer (CaP) growth and metastasis using two complementary approaches, i.e., the antisense approach and the use of a HAase inhibitor, VERSA-TL 502. We also plan to examine the mechanism by which HYAL1 regulates CaP cell growth and metastasis.

The majority of the newly diagnosed prostate cancer (CaP) patients have clinically organ-confined disease. However, limited knowledge about which CaP is likely to progress, as well as, when it will recur severely impedes individualized selection of therapy and subsequent prediction of outcome (1-5). Advances in tumor biology have unraveled genes and their products that closely associate and function in CaP growth, metastasis and angiogenesis (1,6-8). Some of the molecules may serve as accurate prognostic indicators. Moreover, treatment modalities that target the functions of these molecules could effectively control CaP progression. In preliminary studies we identified that HA and HYAL1 type HAase may be such markers. HA is a glycosaminoglycan that is made up of repeating disaccharide units, D-glucuronic acid and N-acetyl-D-glucosamine. It is abundantly present in tissues and tissue fluids. In addition to its structural role, HA regulates several cellular processes (9-10). Concentrations of HA are elevated in several cancers including those of colon, breast and in bladder (11-19). In a published report that we had presented as the preliminary evidence at the time of submission of this funded project, we demonstrated that HA levels are 4-8-fold elevated in CaP tissues, when compared to the normal prostate and benign prostatic hyperplasia tissues (20). Immunohistochemical analysis demonstrated that HA present in CaP tissues mostly localizes to the tumor-associated stroma. Small fragments of HA are known to be anigogenic and we showed the presence of such angiogenic HA fragments in high-grade CaP tissues.

HAase is an endoglycosidase that cleaves internal β-N-acetyl-D-glucosaminic linkages in the HA polymer, yielding HA fragments (21). At present 6 HAase genes have been identified, which cluster into two tightly linked triplets on chromosomes 3p21.3 (HYAL1, HYAL2 and HYAL3) and 7q31.3 (HYAL4, PH20, HYALp1) (22). Using RT-PCR, cDNA cloning/sequencing, cell-culture, immunoblotting and pH activity profile studies, we confirmed that HYAL1 is the HAase that is expressed in CaP tissues and it is secreted by CaP cells (23). We also showed that HAase levels are elevated in CaP tissues when compared to the levels in NAP and BPH tissues. Furthermore, the increase in HAase levels correlates with CaP progression (metastatic > high-grade >> low-grade (Gleason 5/6) > NAP/BPH) (23). By immunohistochemical analysis, which was presented as the preliminary evidence during the submission of this funded project, we showed that the HYAL1 type HAase is exclusively expressed in CaP cells. Based on our immunoshistochemical studies we hypothesized that HA and HYAL1 may be potentially accurate prognostic indicators for CaP. To investigate the function of HYAL1 we had stablely transfected DU145 cells with full-length HYAL1 cDNA in the sense and antisense orientation and planned to study the behavior of DU145, as well as, PC3-ML transfectants both *in vitro* and *in vivo*. In addition, we had identified a HAase inhibitor, VERSA-TL502 (24). This inhibitor inhibits the HAase activity secreted by CaP cells (IC_{50} 2 μg/ml). We had hypothesized that VERSA-TL 502 may also inhibit CaP cell growth and invasive behavior, both *in vitro* and *in vivo*.

The following is a succinct report of the progress in achieving our objectives in the two years of the three-year project.

B. (BODY): Progress related to Aim 1: To correlate HA and HYAL1 staining intensity and its pattern in CaP tissues with clinical outcome.

Rationale and background: Two studies were conducted under this aim. The first study involved evaluation of the prognostic potential of HA and HYAL1 to predict biochemical recurrence within 64 months (i.e., within 5-years). Our results show that HYAL1 either alone or in combination with HA is an independent predictor of biochemical recurrence in CaP patients. This study was presented in detail in the first year's progress report and has been published (25; Appendix 1). Out of the 70 patients that were included in that study follow-up of $\geq$72 months (72 to 131 months) was available on 66 patients. In the study described below, we compared the prognostic ability of HA and HYAL1 with two other potential prognostic indicators of CaP (i.e., CD44v6 and microvessel density (MVD)) to predict biochemical recurrence up to 131 months.

We chose to compare CD44v6 and MVD with HA and HYAL1 since all of these molecules are biologically related. For example, CD44 denotes a family of cell surface transmembrane glycoproteins which serve as the cell surface receptor for HA (26,27). Alternative splicing of CD44 mRNA in 10 of the 20 exons generates several variant CD44 isoforms (27,28). The correlation between tumor progression and CD44s and/or its isoforms is controversial. We and others have shown that the androgen insensitive CaP line PC-3 and primary PCa cells express CD44s and CD44 variants (e.g., CD44v3 and CD44v6), however, the androgen sensitive poorly metastatic line LNCaP does not express CD44 (29-31). Contrary to these findings, it has been shown that the over-expression of CD44v6 in a rat CaP line decreases metastasis (32). Recently, Ekici et al showed that decreased expression of CD44v6 could be a predictor of poor prognosis in clinically localized CaP (33). Aaltomaa et al also reported similar results (34).

Angiogenesis is an essential process for tumor growth and metastasis (35-37). As discussed above, degradation of HA by HAase generates angiogenic HA fragments. Clinical significance of angiogenesis, measured as microvessel density (MVD), has been demonstrated for several tumor types, including, gastrointestinal, breast, bladder and renal cell carcinomas (38-41). Studies that compared various endothelial cell markers (i.e., CD31, CD34 and Factor VIII) have shown that CD34 is a sensitive endothelial cell marker for measuring MVD (42,43). At the present time, the clinical significance of MVD, as an independent predictor of pathological stage and recurrence in PCa remains controversial (42-45).

Since HYAL1 degrades HA and generates angiogenic fragments and CD44 acts as a cell-surface receptor for both HA and HA fragments, it is interesting to examine whether these biologically linked molecules are accurate prognostic indicators for PCa, and whether they influence each other's prognostic capabilities.

B.2 Experimental procedures:

Specimens and study individuals: We initially chose 150 archival specimens from CaP patients who underwent retropubic prostatectomy for clinically localized CaP between 1992 and 1996. Out of these 150 patients, on 66 patients, a minimum available follow-up of 72 months was available. Of the 66 patients, 25 patients had biochemical or clinical recurrence before 72 months (mean time to recurrence: 21.3 months; range: 3 to 61 months), and 41 patients were free of disease recurrence (mean follow-up: 103 months; range: 72 - 131 months). Biochemical recurrence was defined as a PSA level $\geq$ 0.4 ng/ml in 2 successive measurements after the operation, in which case the first date of elevated PSA level was considered as the

date of failure. The patient characteristics with respect to age, preoperative PSA, and tumor (i.e., Gleason sum, stage, margin, extraprostatic extension (EPE) and seminal vesicle invasion) are shown in Table 1.

Table 1: Distribution of pre- and post-operative parameters among study patients. Note that biochemical recurrence with post-operative PSA levels ≥ 0.4 ng/ml within 72 months was used a cut point for defining progression. Thus, any CaP patient who showed a biochemical recurrence within 72 months was included in the progressed category.

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 Mean: 65.1	Median: 9.0 Mean: 14.04	T1c: 10 T2a: 5 T2b: 10	6 = 2 7 = 14 8 = 6 9 = 3	(+) = 21 (-) = 4	(+)=18 (-)=7	(+) = 14 (-) = 11
No biochemical or clinical recurrence (n = 41)	Median: 65 Mean: 62.98	Median: 6 Mean: 8.1	T1c: 22 T2a: 5 T2b: 14	5 = 7 6 = 9 7 = 20 8 = 5	(+) = 4 (-) = 37	(+)=9 (-)=32	(+) = 3 (-) = 38

HA and HYAL1 staining: IHC localization of HA and HYAL1 in CaP tissues was carried out as described previously (20). For all specimens, paraffin-embedded blocks containing CaP tissues representing the majority of the Gleason sum were selected by the study's pathologist (Dr. Francisco Civantos). The blocks were cut into 3-μm thick sections and placed on positively charged slides. The specimens were deparafinized, 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 (20). The specificity of HA staining was established as described previously (20). Following incubation with the HA-binding protein, the slides were washed in phosphate buffered saline (PBS) and sequentially incubated with streptavidin peroxidase at room temperature for 30 min and 3,3'-diaminobenzidine (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 4^0 C for 16 hr. Rabbit polyclonal anti-HYAL1 IgG was generated against a peptide sequence present in HYAL1 protein (amino acids 321 to 338) and its specificity for IHC was confirmed as described previously (20, 25). Following incubation with anti-HYAL1 IgG, the slides were washed in PBS and incubated with a linking solution containing a biotinylated goat anti-rabbit IgG (Dako LSAB kit) 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 for HA and HYAL1: Two readers 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 HYAL-1 was graded as 0 (no staining), 1+, 2+, and 3+. For HA staining, both the tumor-associated stroma and tumor cells

were graded in each slide. 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+ and 2+ staining, and high-grade staining included 3+ intensity. 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-HYAL-1 staining, a positive result was indicated only when both HA (stromal, tumor cells, or both) and HYAL-1 staining intensities were of high grade. Any other combination was considered negative. All slides were reviewed out of order to prevent direct comparison of individual cases for HA and HYAL1.

CD34 staining: Following the antigen retrieval step (as described above), the slides were incubated with a mouse monoclonal anti-human hematopoietic progenitor cell CD34 antibody (dilution of 1:20; DAKO, Denmark) at 4°C for 15 hours. The slides were then incubated with a biotinylated anti-mouse antibody and an avidin-peroxidase conjugate solution (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA). To visualize peroxidase binding sites, the slides were incubated with a 3,3'-diaminobenzidine (DAB) chromogen substrate solution (Dako Laboratories) for 10 minutes. The slides were counterstained with hematoxylin, dehydrated and mounted.

The method described by Weidner et al (42) was used for scoring of the microvessels stained with CD34. The area of the highest MVD in each tissue specimen was localized under 40X magnification and was designated as "hot spot". The microvessels in the hot spots were counted under 400X-magnification. Any vessel with lumen and endothelial cell or endothelial cell cluster stained positively for CD34 was considered to be a single countable microvessel. MVD count was defined as the mean value of the counts obtained in 3 separate, contiguous but not overlapping areas within the hot spot. A cutoff value was determined using the receiver operating characteristic (ROC) curve and according to this value two groups of low and high MVD were assigned. Microvessels were examined and counted by the three readers (S.E., V.B.L. and W.H.C.) independently and without the knowledge of the clinical and pathological status of the patients. The sections were reviewed out of order to prevent direct comparison of individual cases for CD34.

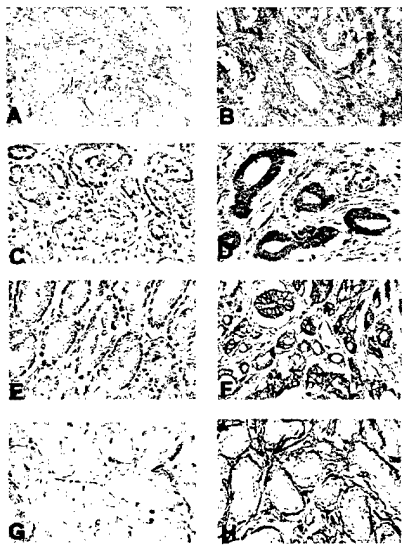
CD44v6 staining: Following antigen retrieval, the slides were incubated with a mouse monoclonal anti-human CD44v6 antibody (dilution of 1:50; Bender Med systems, Vienna, Austria) at 4°C for 15 hours. The sections were then incubated with a biotinylated secondary antibody and an avidin-peroxidase conjugate solution (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA). To visualize peroxidase binding sites, the slides were incubated with a 3,3'-diaminobenzidine (DAB) chromogen substrate solution (Dako Laboratories) for 10 minutes. The slides were counterstained with hematoxylin, dehydrated and mounted.

The slides for CD44v6 were scored as described by Ekici et al (33). All sections included normal prostate tissue and/or benign prostatic hyperplasia glands as internal controls. Intensity of staining was graded as 0 for no staining, 1 for weak intensity, 2 for moderate intensity and 3 for strong intensity. A combined staining score based on an estimate of the percentage of tumor cells stained and the intensity of staining was developed. The areas of tumor cells stained with maximum intensity (primary area) and the other tumor cells stained with lesser intensity (secondary area) were determined in percentage values. The combined score is obtained by adding the scores of the primary and secondary areas. Staining intensities were examined and

scored by two readers (S.E. and V.B.L.), independently and in a blinded fashion. A cutoff value was determined from the ROC curve and according to this value two groups of low and high CD44v6 staining were assigned.

Statistical Analysis:

The inter-assay variability regarding staining intensity was determined by Pearson's correlation analysis. The Spearman's bivariate correlation coefficients were 0.85, 0.9, 0.98 and 0.95 for HA, HYAL1, CD34 and CD44v6 staining, respectively. For all the markers, a high-grade staining was considered as a true positive if the patient had biochemical recurrence. Consequently, low-grade staining was considered as a true negative, if the patient had no biochemical recurrence. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for HA, HYAL1, HA-HYAL1, CD34 and CD44v6 staining inferences were calculated using the 2 x 2 contingency table (high-grade/low-grade staining and progressed/non-progressed CaP patients) at 72, 84, 100 and 112 month cut-off limits. For CD44v6 and MVD, Receiver Operating Characteristic curves were developed for determining the optimal cut-off limits that yielded the best possible sensitivity and specificity values. The cut-off limits for CD44v6 and MVD were 180 and 41, respectively. The sensitivity is defined as: true positive (i.e., No. of recurred patients predicted by a marker) ÷ total no. of recurred patients. Specificity: true negatives (i.e., No. of non-recurred patients predicted by a marker) ÷ total no. of non-recurred patients. Accuracy: No. of True positive + No. of true negatives ÷ total no. of CaP patients in the study. PPV: No. of true positive ÷ No. of true positive + No. of false positive. NPV: No. of true negative ÷ No. of true negative + No. of false negative. The data on various, biochemical, surgical, and pathologic parameters, as well as HA, HYAL1, HA-HYAL1, CD34 and CD44v6 staining inferences, were analyzed by Cox Proportional Hazard model, using single variable analysis (univariate analysis) or step-wise selection analysis. Stratified Kaplan-Meier analyses were performed on the variables that were found to be significant in the multivariate Cox Proportional Hazard model. Statistical analysis was carried out under the direction of project's statistician, Dr. Robert Duncan, using the SAS Software Program (version 8.02; SAS Institute, Cary, NC).



Localization of HA, HYAL1, CD44v6 and MVD in CaP tissues: A non-recurred patient (panels A, C, E, G) and a recurred patient (panels B, D, F, H). Panels A and B: HA; Panels C and D: HYAL1; Panels E and F: CD44v6; Panels G and H: MVD

Results:

Immunohistochemistry of the tissue markers:

Fig. 1 shows immunohistochemical localization of HA, HYAL1, CD44v6 and MVD in 2 Gleason 7 PCa specimens, one each from a non-recurred (panels A, C, E, G) and a recurred (panels B, D, F, H) patient, respectively. As shown in Fig. 1 panel A, very little HA staining is seen in PCa tissue from a patient who did not progress within 72 months. Among the 41 PCa specimens from non-recurred patients, 25 showed low-grade staining. Panel B shows high-grade HA staining in PCa specimen from a patient who had biochemical recurrence in < 72 months (median time to recurrence: 19 months; mean time to recurrence: 21.3 months). The HA staining is seen mainly in tumor-associated stroma.

However, high-grade HA staining was also seen in tumor cells in 8 out of 25 patients who had biochemical recurrence. Out of the 25 patients who had recurred, 24 showed high-grade HA staining.

As shown in Fig. 1 panel C, little HYAL1 staining is seen in the PCa tissue from a non-recurred patient. Out of the 41 non-recurred patients, PCa specimens from 33 had low-grade staining. In the PCa specimen from a patient who later recurred, high-grade HYAL1 staining is seen (Fig. 1, panel D). The HYAL1 expression is seen exclusively in tumor cells. Out of the 25 patients who recurred within 72 months, 21 had high-grade HYAL1 staining. Contrary to some earlier reports (33,34), low-grade CD44v6 staining is observed in the PCa specimen from a non-recurred patient (Fig. 1, panel E) and high-grade CD44v6 staining is observed in the PCa tissue from a recurred patient (Fig. 1, panel F). CD44v6 staining is mostly associated with the plasma membrane of tumor cells. We also observed CD44v6 in non-neoplastic epithelial cells in normal prostate and benign prostatic hyperplasia glands. However, the staining intensity of CD44v6 in non-neoplastic cells was less than that in tumor cells. Using a cut-off limit of 180 on the scoring scale, 23 out of 41 PCa specimens from non-recurred patients showed low-grade staining, whereas, out of the 25 patients who recurred, 17 showed high-grade staining.

As shown in Fig. 1 panel G, MVD is low in the PCa tissue from a non-recurred patient. As determined from the Receiver Operating Characteristic curve, a cut-off limit of 41 was set to score low or high MVD. Out of the 41 non-recurred patients, PCa tissues from 25 patients had low MVD. However, the MVD was high in 19 out of 25 PCa tissues obtained form patients who had a recurrence. Fig. 1 panel H shows high MVD in the PCa specimen from a patient who later recurred.

Determination of sensitivity, specificity, accuracy: We determined sensitivity, specificity, accuracy HA, HYAL1, combined HA-HYAL1, CD44v6, and MVD at 72-, 84-, 100- and 112-months of follow-up. Data on 72 and 112 months is shown here (For details please see Appendix 2; ref. 46). As shown in Table 2, the sensitivity of HA, HYAL1, combined HA-HYAL1, CD44v6, and MVD for predicting CaP recurrence is 96%, 84%, 84%, 76% and 68%, respectively within 72 months. The specificity, of HA (61%), CD44v6 (56.1%), and MVD (61%) was lower than that of HYAL1 (80.5%) and combined HA-HYAL1 (87.8%). The accuracy of the

Parameter	**HA (%)** 72 months	112 months	**HYAL1 (%)** 72 months	112 months	**HA-** 72 months	**HYAL1 (%)** 112 months
Sensitivity	96	92.6	84	85.2	84	81.5
Specificity	61 (25/41)	80.6	80.5	94.4	87.8	94.4
Accuracy	74.2	91.1	81.8	88.9	86.4	86.7
Parameters	**CD44v6 (%)** 72 months	112 months	**MVD (%)** 72 months	112 months		
Sensitivity	72 months	112 months	72 months	112 months		
Specificity	76	77.8	68	62.9		
Accuracy	61	77.8	56.1	61.1		

Table 2: 72 and 112 months were used a cut point for determining biochemical recurrence.

HA-HYAL1 (86.4%) was the highest, followed by HYAL1 (81.8%), HA (74.2%), MVD (66.7%), and CD44v6 (57.6%). Follow-up information of ≥112 month was available on 45 patients (mean follow-up 121 months; median 120.2; range 112 – 131 months). At 112 month, the sensitivity of HA, HYAL1, HA-HYAL1, MVD and CD44v6 was 92.6%, 85.2%, 81.5%, 77.8% and 62.9% respectively. At final analysis, both HYAL1 and combined HA-HYAL1 had the best specificity (94.4%, 94.4%) and accuracy (88.9%, 86.7%), followed by HA, MVD and CD44v6 (Table 2).

Evaluation of the prognostic capabilities of pre-operative and post-operative parameters and histological markers:

Univariate analysis:

Since the study patients in this cohort had variable follow-up between 72 and 131 months, we used Cox Proportional Hazard model and single parameter analysis to determine the prognostic significance of each of the pre-operative (i.e., age, PSA and clinical stage) and post-operative parameters (i.e., Gleason sum, margin +/-, EPE +/-, seminal vesicle invasion +/-), as well as, staining inferences of HA, HYAL1, combined HA-HYAL1, CD44v6 and MVD. In the univariate analysis, age (p = 0.5104; hazard ratio = 1.019), clinical stage (p = 0.2683, hazard ratio = 1.2620) and CD44v6 staining (p = 0.131 hazard ratio = 1.826) were not significant in predicting biochemical recurrence. However, pre-operative PSA (p = 0.0006, hazard ratio/unit PSA change = 1.048), Gleason sum overall (p = 0.0002; hazard ratio = 2.5), margin status (p = 0.0003; hazard ratio = 4.5), EPE (p<0.0001; hazard ratio = 12.781), seminal vesicle invasion (p<0.0001; hazard ratio = 6.56), HA staining (p=0.0008; hazard ratio = 12.091), HYAL1 staining (p<0.0001; hazard ratio = 13.192), HA-HYAL1 staining (p<0.0001; hazard ratio = 10.749) and MVD (p = 0.0015; hazard ratio = 4.36) significantly predicted biochemical recurrence. (Please see Appendix 2 for details, ref. 46).

Parameter	P Value	Hazard Ratio
PSA	< 0.0001*	1.068
EPE	0.0016*	6.222
HYAL1	0.0009*	8.196
HA-HYAL1	0.0021*	5.191

Table 3: HA and HYAL1 or HA-HYAL1were included in the multivariate analysis; *: Statistically significant

Multivariate analysis:

To determine the smallest number of variables that could jointly predict biochemical recurrence in this cohort of study patients, we used Cox Proportional Hazard model and step-wise selection analysis. When age, pre-operative PSA, clinical stage, Gleason sum (overall or ≥ 7), EPE, seminal vesicle invasion and staining inferences of HA, HYAL1, CD44v6, and MVD were included in the model, only pre-operative PSA (p < 0.0001, hazard ratio/unit PSA change = 1.086), EPE (p = 0.0016, hazard ratio = 6.222) and HYAL1 (p = 0.0009, hazard ratio = 8.1896) reached statistical significance in predicting biochemical recurrence (Table 3).

The inclusion of the combined HA-HYAL1 staining inference instead of HA and HYAL1 staining inferences, in the multiple regression model again showed that pre-operative PSA (p = 0.0002, hazard ratio/unit PSA change 1.077), EPE (p = 0.0009, hazard ratio = 6.906) and HA-HYAL1 (p = 0.0021, hazard ratio = 5.191) were significant in predicting biochemical recurrence (Table 3 B). None of the other pre-operative (PSA, clinical stage) and post-operative parameters (Gleason sum and seminal veicle) or CD44v6 and MVD staining inferences reached statistical significance in the multivariate model (P > 0.05, in each case).

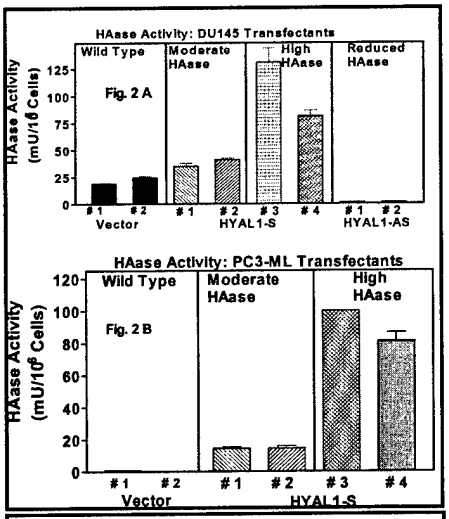
Summary: The results, which we have obtained, demonstrate that HYAL1 and HA-HYAL1 are independent prognostic indicators for predicting biochemical recurrence of CaP. An additional point that deserves attention is that in this study we had long follow-up on each patient (minimum follow-up 72 months), which was sufficient to detect any biochemical recurrence. This long follow-up makes a strong case that HYAL1 and HA-HYAL1 are potentially useful prognostic indicators for CaP.

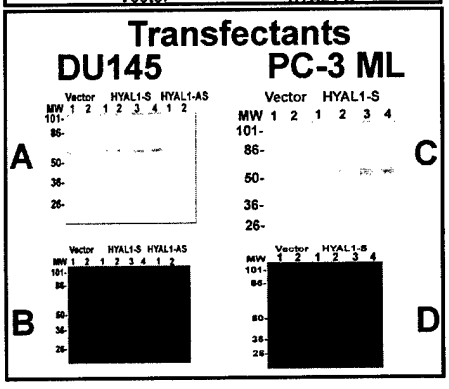
C. (BODY): Progress related to Aim 2: To evaluate the effect of HYAL1 inhibition on CaP growth and metastasis.

C. 1. Rationale and background: When we submitted the above referenced grant we had generated DU145 transfectants that either over produced (HYAL1 sense) or under produced

(HYAL1- antisense) HYAL1-type HAase. Contrary to our hypothesis that HYAL1 enhances CaP growth, when we injected these transfectants in athymic nude mice to study the effect of HYAL1 on tumor growth, we found that both the HYAL1-sense and HYAL1-antisense transfectants did not generate palpable tumors in 30 days, whereas, the vector only transfectants (i.e., wild type) generated palpable tumors in 7 days. As a result, we generated DU145 transfectants again and this time we selected two different types of HYAL1-sense transfectant clones: 1. Moderately HYAL1 over-producing 2. Highly HAase over-producing. Since PC3-ML cells express little HYAL1, we generated only HYAL1-sense transfectants and again selected moderately HAase-overproducing and highly HAase-overproducing clones. Both the *in vitro* and *in vivo* analysis of these clones demonstrates that both, the over and under production of HYAL1 decreases CaP growth and its invasive potential. Our experimental strategy and results are discussed below, along with our results on the use of HAase inhibitors.

C.2. Effects of HYAL1 overproduction and under production on CaP growth and invasion:
C.2. a.: Generation of DU145 and PC3-ML transfectants: The entire HYAL1 cDNA coding region (nucleotides 618 – 1925 GenBank # HSU03056) was amplified by RT-PCR analysis as described previously (47). The amplified cDNA was directly cloned into pcDNA 3.1/v5/His-TOPO eukaryotic expression vector, using a TOPO-TA cloning kit (Invitrogen). This vector allows bi-directional cloning of PCR products, i.e., the cDNA has a 50:50 chance of insertion into the vector either in the sense or antisense orientation with respect to the CMV promoter. HYAL1-sense (HYAL1-S) and HYAL1-antisense (HYAL1-AS) constructs were used for transfection studies. Since DU145 cells produce high levels of HAase, we generated both HYAL1-S and HYAL1-AS transfectants. However, for PC3-ML cells, we generated only HYAL1-S transfectants since these produce very little HAase (1-3 mU/10^6 cells). CaP cells (2 x 10^5) were transfected with 5-μg DNA of HYAL1-S, HYAL1-AS or pcDNA3.1/v5-His vector constructs using Effectene™ transfection reagent and a protocol supplied by the manufacturer (Qiagen). The transfectants were selected in geneticin (400-μg/ml for DU145 and 300-μg/ml for PC3-ML). 25 to 30 transfectant clones for each construct were analyzed and data on 2 representative clones are presented.

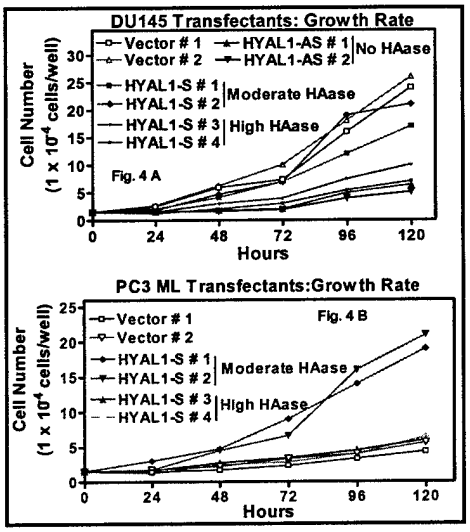




C.2. b. Analysis of HAase secretion by transfectants: The HAase secretion by various transfectants was analyzed using the HAase ELISA-like assay that we have described previously (47, 48). Briefly, microtiter wells coated with 200 μg/ml HA are incubated with different samples (e.g., culture conditioned medium (CM)) in an HAase assay buffer (47,48). Following incubation, the degraded HA is washed off and HA remaining on the wells is detected using a biotinylated HA binding protein and an avidin-biotin detection system. HAase activity (mU/ml) is normalized to total protein (mg/ml) or to cell number.
As shown in Fig 2 A, we isolated 2 types of HYAL1-S transfectants for DU145 cells: 1. Moderately HAase overproducers (clones HYAL1-S # 1 and HYAL1-S # 2) which produce 1.5 – to 2 fold higher HAase than the vector transfectants. 2. High HAase producers (clones HYAL1-S

3 and HYAL1-S # 4). These secrete 5-7.5-fold more HAase activity than the control transfectants. HYAL1-AS transfectants (clones HYAL1-AS # 1 and HYAL1-AS # 2) show > 95% reduction in HAase production. As shown in Fig. 3 A, anti-HYAL1 immunoblot anlaysis and a substrate (HA) gel assay, which detects active HAase protein, show that DU145 vector and HYAL1-S transfectants secrete a ~ 60 kDa enzymatically active protein in their conditioned media (CM). As expected, higher amount of HYAL1 protein is present in the CM of HYAL1-S # 3 and # 4. This protein is not detected in HYAL1-AS transfectants' CM.

As shown in Fig. 2 B, PC3-ML vector transfectants secrete very little HAase activity. However, moderately high HAase producing clones (HYAL1-S # 1 and # 2) secrete 10-fold more HAase activity in their CM and the high HAase producers secrete 80-100-fold more HAase than the vector controls. Immunoblot analysis and substrate (HA)-gel analysis confirm these date (Fig. 3 B).



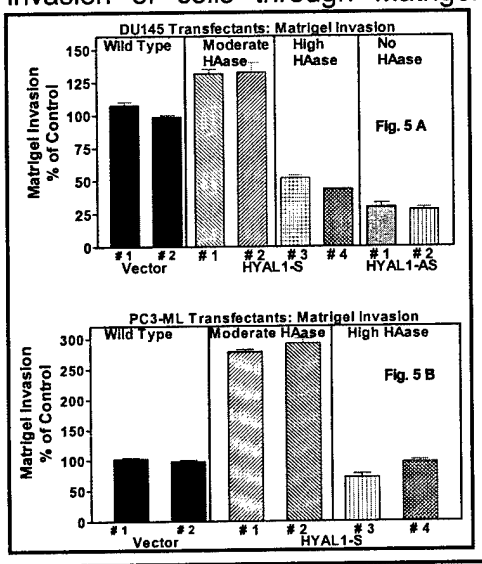
C.2.c. Cell proliferation and cell-cycle anlaysis of transfectants: To determine the doubling time of transfectants, vector, HYAL1-S and HYAL1-AS clones of CaP cells (2 x 10^4 cells/well) were plated on 24-well culture plates in growth medium (RP MI 1640 + 10% FBS plus gentamicin and geneticin). Every 24 hr, for a total of 120 hr, cells were trypsinzed and counted. All counts were done in duplicates and in 3 independent experiments (SEM < 10%). As shown in Fig. 4 A, moderate overproducers (HYAL1-S # 1 and # 2) and vector transfectants grow at a similar rate (doubling time ~ 24 hr). However, the high producers (HYAL1-S # 3 and # 4) grow ~ 3 times slower (doubling time ~ 60- 80 h). Interestingly, HYAL1-AS transfectants also grow ~ 4.5 times slower (doubling time 72-80 h) when compared to the vector transfectants. In case of PC3-ML transfectants, the moderate producers grow much faster (3.5-4.5-fold) than vector transfectants and high HAase producers (Fig. 4 B). It has been previously shown that HYAL1 expression in PC-3 cells does not change the growth rate *in vitro*, however in that study HYAL1-S transfectants were not categorized as moderate and high HAase producers (49).

Name	G0-G1	S	G2-M
Vector # 1	51.3%	47.8%	0.92%
Vector # 2	51.5%	44.9%	3.5%
HYAL1-S # 1	51.5%	47.9%	0.57%
HYAL1-S # 2	48.6%	51.4%	0%
HYAL1-S # 3	60.3%	29.8%	9.9%
HYAL1-S # 4	56.9%	36%	7.03%
HYAl1-AS # 1	53.8%	35.8%	10.4%
HYAL1-AS # 2	56.7%	33.6%	9.7%

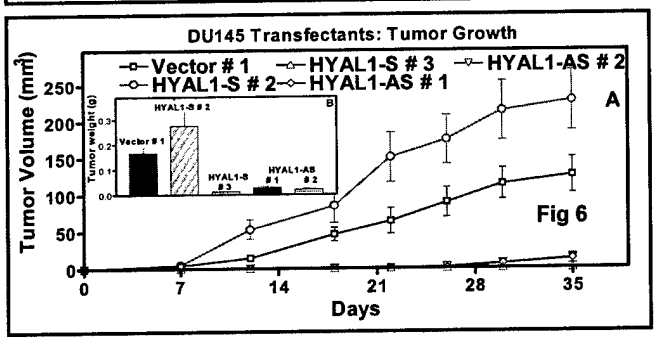
For **cell-cycle analysis** growing cultures of transfectants were lysed in a hypotonic solution containing 0.1% NP40 and propidium iodide. The lysed cells (10^6/ml) were analyzed using an EPICS XL flow cytometer equipped with long pass red filter, FL3 (630 nm). As shown in Table 4, high producers and antisense transfectants of DU145 appear to be blocked in G2-M phase of the cell-cycle, consequently, lower percentage of cells are in the S-phase. Cell-cycle analysis showed that high HAase producing PC3-ML transfectants are also blocked in G2-M phase (data not shown).

C.2.d. Matrigel invasion assay: The membranes in Transwell plates (Costar) were coated with Matrigel (100-μg/cm^2). DU145 or PC3-ML transfectants were plated on the upper chamber (3x10^5 cells/well) in RPMI + ITS (insulin, transferrin, selenium) + gentamicin + geneticin. The

bottom chamber contained growth medium (chemoattractant). Following 48 h incubation, the invasion of cells through Matrigel was quantified using MTT assay (50). To normalize differences in cell proliferation rates among transfectants, the results were calculated as the ratio of cells in the lower chamber to the total number of cells (upper + lower). The invasive activity of vector transfectants was taken as 100%. As shown in Fig. 5 A HYAL1-S # 1 and # 2 clones of DU145 transfectants have similar invasive activity as the vector transfectants. However, both high producers (HYAL1-S # 3 and # 4) and HYAL1-AS transfectants are 50% to 70% less invasive than the vector transfectants. Similarly, HYAL1-S # 1 and # 2 PC3-ML transfectants are 270% to 300% more invasive than the vector and high HAase producers (HYAL1-S # 3 and # 4; Fig 5 B).



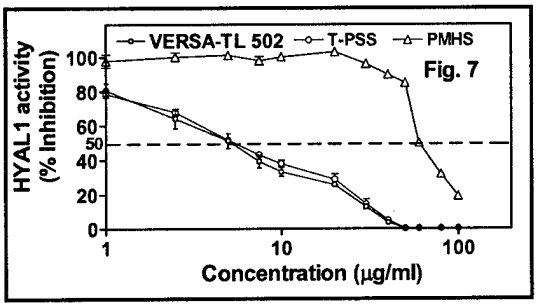
C.2.e.: Analysis of HYAL1 function *in vivo*: Since accurate measurement of tumor growth is possible in an subcutaneous model, we chose s.c. model over the orthotopic implant model. DU145 vector # 1, HYAL1-S # 2, HYAL1-S # 3 and HYAL1-AS # 1 and # 2 transfectant clones ($2x10^6$ cells) were mixed with Matrigel (1:1 v/v) and injected on the dorsal flanks of athymic mice. Six mice were included in each group (1 injection site/mouse). Once the tumor became palpable (in 7-days for vector # 1 and HYAL1-S # 2 clones), tumor size was measured twice weekly. Tumor volume was calculated as length (mm) x width x depth x 0.5236 (51). Mice were euthanized at day 35 and tumors were excised, weighed and fixed for histology. As shown in Fig. 6 A, while vector # 1 and HYAL1-S # 2 tumors grew steadily, palpable tumors were not generated in all 6 mice injected with HYAL1-S # 3. Two animals each in HYAL1-AS # 1 and # 2 groups developed palpable tumors at day 30. Excised tumors showed that HYAL1-S # 2 generated slightly larger tumors (~ 1.6-fold more) than the vector # 1 transfectant (Fig. 6 B). However, the tumor weight of HYAL1-S # 3 and HYAL1-AS # 1 and # 2 transfectants was 16-27-fold less when compared to vector # 1 and HYAL1-S # 2 clones. (Fig. 6 B). Tumor histology revealed that suspected tumor tissues removed from HYAL1-S # 3 and HYAL1-AS (# 1 and # 2) bearing animals consisted mainly of a vascularized and vascularized Matrigel plug (Data not shown).



In summary, the results presented in this section show that while moderate HYAL1 expression (~ 15 – 35 mU/10^6 cells) production promotes CaP cell growth (both *in vitro* and *in vivo*) and their invasive potential, very high HYAL1 production (≥80 mU/10^6 cells), as well as, little HAase production (< 3 Mu/10^6 cells) severely decreases CaP growth and its invasive potential. Therefore, perturbation of this finely regulated balance of HAase production by tumor cells may a new treatment target to control CaP growth and progression.

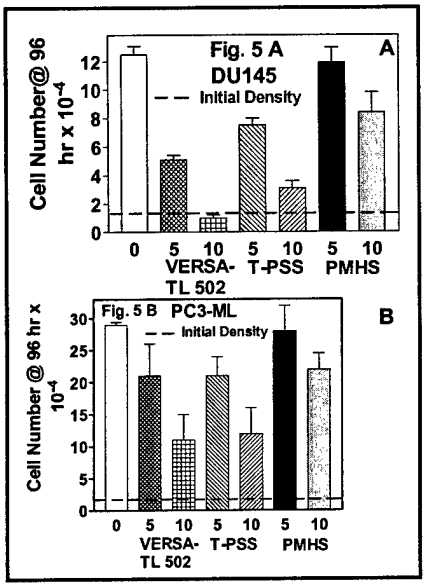
Effect of HAase inhibitors on the HAase activity secreted in DU145 culture conditioned media: In addition to VERSA-TL 502 that we had mentioned in our proposal we obtained 2 more HAase inhibitors namely T-PSS (Polystyrene sulfonate) and PMHS (Polymethylene hydroquinone sulfonate). Both these inhibitors were kindly provided by Drs. Drs. Anderson and

Zaneveld. According to Drs. Zaneveld, T-PSS is as good an inhibitor as VERSA-TL 502 to inhibit the testicular HAase, however, PMHS is a weaker inhibitor.



We compared the ability of these inhibitors to block the HYAL1 type HAase activity secreted in the conditioned media using the HAase ELISA-like assay, described in section C.2.a.

As shown in Fig. 7 both VERSA-TL 502 and T-PSS caused a dose-dependent inhibition of HAase activity present in the conditioned medium of DU145 cells (IC_{50} 5.2 µg/ml). However, PMHS was a weak inhibitor of HAase activity (IC_{50} 60 µg/ml).



C.2 Effect of HAase inhibitors on the growth of DU145 and PC3-ML cells. To test the effect of HAase inhibitors on CaP cell growth, DU145 and PC3-ML cells (1.5×10^4 cells/well) were plated on 24-well plates in growth medium in the presence of various concentrations (0-20 µg/ml) of VERSA-TL 502, T-PSS or PMHS. Every 24-hr for 96-hr, cells were stained with trypan blue and counted. As shown in Fig. 5 A and B HAase inhibitors inhibit growth of both DU145 and PC3-ML cells. Furthermore, among the 3 inhibitors, VERSA-TL 502 was the most effective. At 5 µg/ml, IC_{50} for HAase activity, the growth inhibition by VERSA-TL 502 was 61% in DU145 and 30% in PC3-ML cells, when compared to T-PSS (40% DU145 and PMHS (5% for DU145). The reason why PC3-ML cells are less sensitive to HAase inhibitors is most likely that they have low HYAL1 expression.

Currently, we are testing the effect of these HAase inhibitors on the invasive properties of CaP cells using the Matrigel™ invasion assay (28).

In summary, the results that we have obtained so far demonstrate that HAase inhibitors are able to inhibit the growth of CaP cells. But more interestingly, the growth inhibitory activity of HAase inhibitors correlates with the HAase inhibitory activity of these inhibitors. For example, VERSA-TL 502 is a potent inhibitor of HAase activity secreted by CaP cells and it is an effective inhibitor CaP cell proliferation. On the contrary, PMHS, which is a weak inhibitor of HAase activity, causes very little growth inhibition.

D. (BODY) Experiments in progress

Examination of the *in vivo* behavior of PC3-ML transfectants: We have implanted PC3-ML vector (# 1, # 2 and HYAL1-S (clones # 1, #2, #3, and # 4) transfectants in athymic mice using the protocol described in section C.2.e. We will be injecting 6 athymic mice per transfectant group, on January 15th and monitor tumor growth as described in section C.2.e..

Examination of the effect of VERSA-TL 502 on CaP growth: We are in the planning process to study the effect of VERSA-TL 502 on the growth of DU145 and PC3-ML cells in xenografts.

These experiments will begin in March, because at that time we will already have results of the PC3-ML transfectnat xenograft experiment.

Microarray analysis of DU145 and PC3-ML transfectants: We have isolated total RNA from each transfectant clone of DU145 (vector clones 1 and 2, HYAL1-S clones 1 to 4 and HYAL1-AS clones 1 and 2) for microarray analysis. Microarray analysis will be performed on these RNA samples in a pair-wise fashion (e.g., vector # 1 and HYAL1-S # 3) in our university's microarray facility. Similar experiments will be conducted on PC3-ML transfectants.

2 Key Research accomplishments:

A. Establishment of HYAL1 and combined HA-HYAL1 staining inferences as potentially accurate predictors of biochemical recurrence. The studies presented here are **the first**, which demonstrate the prognostic potential of HYAL1 in any type of cancer.

B. Demonstration that CaP cells finely regulate HYAL1 expression, which in turn, is responsible for increased CaP growth and invasive activity. Overproduction of HYAL1 or blocking its expression may be new avenues for controlling CaP growth and progression.

C. Synthetic HAase inhibitors inhibit HAase activity secreted by CaP cells and also inhibit the growth of CaP cells. The growth inhibitory activity of HAase inhibitors correlates with their ability to inhibit HAase activity.

3. Reportable outcomes:

A. Database of CaP patients with long-term follow-up (72 – 131 months).

B. Generation of HYAL1-sense (both moderately producing and overproducing) and HYAL1-antisense (under producing) stable cell lines of DU145 and PC3-ML cells.

C. Identification of HAase inhibitors that may be used for controlling CaP growth.

D. Publications: 1. Lokeshwar, V.B. Schroeder, G.L., Carey, R.I. Soloway, M.S., Iida, N. Regulation of hyaluronidase activity by alternative mRNA splicing. J. Biol. Chem. 2002.
2. Posey, J.T., Soloway, M.S., Ekici, S., Sofer, M., Civantos, F., Duncan, R.C., Lokeshwar, V.B. Evaluation of the prognostic potential of hyaluronic acid and hyaluronidase (HYAL1) for prostate cancer. Cancer Res. 63: 2638-2644, 2003.
3. Ekici S., Cerwinka, W.H., Duncan, R. C. Gomez, P., Civantos, F., Soloway, M.S., Lokeshwar, V.B. Comparison of the prognostic potential of hyaluronic acid, hyaluronidase (HYALI-1), CD44v6 and Microvessel density for prostate cancer (Submitted to Int. J. Cancer, 2004).
4. Lokeshwar, V.B., Isoyama, T., Thwaites, F., Selzer, M.G., Carey, R.I. Schroeder, G.L. Differential selectivity of hyaluronidase inhibitors towards acidic and basic hyaluronidases (Submitted to J. Biol. Chem. 2004).

E. Abstracts:

1. Lokeshwar, V.B.*, Posey, J.T., Schroeder, G.L. (2002) HA and HAase in prostate cancer: molecular markers with function. Fourth International Innovators in Urology Meeting, Miami, Florida. Prostate cancer and Prostatic Diseases 5: suppl. 1., S19.

2. Lokeshwar, V.B.*, Posey, J.T., Schroeder, G.L. (2002) HA and HAase in prostate cancer: molecular markers with function. Sylvester Comprehensive Cancer Center Poster Presentation (Annual Zubrad Lecture/Poster Meeting; First Prize in Faculty category).

3. Ekici S., Cerwinka, W.H., Duncan, R. C. Gomez, P., Civantos, F., Soloway, M.S., Lokeshwar, V.B. Comparison of the prognostic potential of hyaluronic acid, hyaluronidase (HYALI-1), CD44v6 and Microvessel density for prostate cancer (AUA 2004; Accepted for Poster Discussion session).

F. Patents: No patents were filed or issued.

G. Clinical translational research: No clinical trials were undertaken.

H. Personnel and training provided: This award has allowed the P.I. to provide research training to two attending urologists. Dr. Sinan Ekici was a fellow of the Turkish Research Council and worked on Aim 1 of the project. Dr. Tadahiro Isoyama is currently working in the laboratory on work related to Aims 2 and 3. In addition, the award has allowed the P.I. to provide research training to a total of 4 urology residents in the Department of Urology at University of Miami. In addition to the trainees, a pathologist, a research associate a statistician and the P.I. worked on this project. One clinical fellow in the department of urology, together with the collaborating urologist, provided the clinical information.

4. Conclusions: Results derived from the experiments performed under Aim 1, demonstrate that HYAL1 and HA-HYAL1 are sensitive and specific markers for predicting biochemical recurrence for CaP patients who undergo radical prostatectomy. The multivariate analysis and long-term follow-up information establishes **for the first time**, the independent prognostic potential of HYAL1 type HAase for any type of cancer, in general, and for CaP in particular. The experiments conducted under Aim 2, demonstrate that, both the over and under production of HAase severely inhibit CaP growth and its invasive potential. Thus, both excess HAase and anti-HAase therapy may be useful in controlling CaP growth and progression.

5. REFERENCES:

1. Pettaway, C.A. Prognostic markers in clinically localized prostate cancer. Tech. Urol., 4: 35-42, 1998.
2. Blute, M.L. Bergstralh, E.J., Iocca, A., Scherer, B., Zincke, H. Use of Gleason score, prostate specific antigenm seminal vesicle and margin status to predict biochemical failure after radical prostatectomy. J. Urol., 165: 119-125, 2001.
3. Muzzonigro, G., Galosi, A.B. Biological selection criteria for radical prostatectomy. Ann N Y Acad Sci. 963:204-212, 2002.
4. Small, E.J., Roach M 3rd. Prostate-specific antigen in prostate cancer: a case study in the development of a tumor marker to monitor recurrence and assess response. Semin Oncol. 29: 264-73, 2002.
5. Palisaan, R.J., Graefen, M., Karakiewicz, P.I., Hammerer, P.G., Huland, E., Haese, A., Fernandez, S., Erbersdobler, A., Henke, R.P., Huland, H. Assessment of clinical and pathologic characteristics predisposing to disease recurrence following radical prostatectomy in men with pathologically organ-confined prostate cancer. Eur Urol. 41:155-161, 2002.
6. Isaacs W, De Marzo A, Nelson W. Focus on prostate cancer. Cancer Cell. 2:113-116, 2002.
7. Muzzonigro, G., Galosi, A.B. Biological selection criteria for radical prostatectomy. Ann N Y Acad Sci. 963:204-212, 2002.

8. Partin, A.W., Murphy, G.P., Brawer, M.K. Report on Prostate Cancer Tumor Marker Workshop 1999. Cancer, 88: 955- 963, 2000.
9.Tool, BP. Hyaluronan is not just a gloo! J. Clin. Invest. 106: 335-336, 2000.
10. Delpech, B., Girard, N., Bertrand, P., Courel, N.M., Chauzy, C., Delpech, A. Hyaluronan: Fundamental principles and applications in cancer. J. Intern. Med. 242: 41-48, 1997.
11.Hautmann, S.H., Lokeshwar, V.B., Schroeder, G.L., Civantos, F., Duncan, R.C., Friedrich, M.G., Soloway, M.S. Elevated tissue expression of hyaluronic acid and hyaluronidase validate HA-HAase urine test for bladder cancer. J. Urol., 165: 2068-2074, 2000.
12. Setala, L.P., Tammi, M.I., Tammi, R.H., Eskelin, M.J., Lipponen, P.R., Argen, U.M., Parkkinen, J., Alhava, E.M., Kosma, V.M., Hyaluronan expression in gastric cancer cells is associated with local and nodal spread and reduced survival rate. Br. J. Cancer, 79: 1133-1138, 1999.
13. Auvinen, P., Tammi, R., Parkkinen, J., Tammi, M., Agren, U., Johansson, R., Hirvikoski, P., Eskelinen, M., Kosma, V.M., Hyaluronan in peritumoral stroma and malignant cells associates with breast cancer spreading and predicts survival. Am. J. Pathol., 156: 529-536, 2000.
14. Pirinen, R., Tammi, R., Tammi, M., Hirvikoski, P., Parkkinen, J.J., Johansson, R. Prognostic value of hyaluronan expression in non-small-lung cancer: increased stromal expression indicates unfavorable outcome in patients with adenocarcinoma. Int J Cancer 95: 12-27, 2001.
15. Knudson, W. Tumor associated hyaluronan: providing an extracellular matrix that facilitates invasion. Am. J. Pathol., 148: 1721-1726, 1996.
16. Ropponen, K., Tammi, M., Parkkinen, J., Eskelinen, M., Tammi, R., lipponen, P., Argen, V., Alhava, E., Kosma, V.M. Tumor-associated hyaluronan as an unfavorable prognostic factor in colorectal cancer. Cancer Res., 58:342-347, 1998.
17. Lipponen, P., Aaltomaa, S., Tammi, R., Tammi, M., Agren, U., Kosma, V.M. High stromal hyaluronan level is associated with poor differentiation and metastasis in prostate cancer. Eur. J. Cancer 37: 849-856, 2001.
18. Lokeshwar, V.B., Obek, C., Pham, H.T., Wei, D.C., Young, M.J., Duncan, R.C, Soloway, M.S., Block, N.L. Urinary hyaluronic acid and hyaluronidase: Markers for bladder cancer detection and evaluation of grade. J. Urol., 163: 348-356, 2000.
19. Lokeshwar, V.B., Obek, C., Soloway, M.S., Block, N.L. Tumor-associated hyaluronic acid: A new sensitive and specific urine marker for bladder cancer. Cancer Res., 57: 773-777, 1997.
20. Lokeshwar, V.B., Rubinowicz, D., Schroeder, G.L., Forgacs, E., Minna, J.D., Block, N.L. Nadji, M., Lokeshwar, B.L. Stromal and epithelial expression of tumor markers hyaluronic acid and hyaluronidase in prostate cancer. J. Biol. Chem. 276: 11922-11932, 2001.
21. Roden, L., Campbell, P., Fraser, J.R.E., Laurent, T.C., Petroff, H., Thompson, J.N. Enzymatic pathways of hyaluronan catabolism. In: The biology of hyaluronan. Whelan, E, (ed), Wiley, Chichister, Ciba Foundation Symp., 143, pp-60-86, 1989.
22. Csoka, A.B., Frost, G.I., Stern, R. The six hyaluronidase-like genes in the human and mouse genomes. Matrix Biol. 20: 499-508, 2001.
23.Lokeshwar, V.B., Lokeshwar, B.L., Pham, H.T., Block, N.L. Association of hyaluronidase, a matrix-degrading enzyme, with prostate cancer progression. Cancer Res., 56: 651-657, 1996.
24. Anderson, A.R., Feathergill, K., Diao, X., Cooper, M., Kirpatrick, R., Spear, P., Waller, D.P., Chany, C., Doncel, G.E., Herold, B., Zaneveld, L.J.D. Evaluation of poly(styrene-4-sulfonate) as a preventive agent for conception and sexually transmitted diseases. J. Androl., 21: 862-875, 2000.
25. Posey, J.T., Soloway, M.S., Ekici, S., Sofer, M., Civantos, F., Duncan, R.C., Lokeshwar, V.B. Evaluation of the prognostic potential of hyaluronic acid and hyaluronidase (HYAL1) for prostate cancer. Cancer Res. 63: 2638-2644, 2003.
26. Lesley, J, Hascall, VC, Tammi, M, Hyman, R. Hyaluronan binding by cell surface CD44. J Biol Chem. 2000 275: 26967-26975.
27. Cichy, J, Pure, E. The liberation of CD44. J Cell Biol. 2003, 161: 839-843.

28. Naor, D, Nedvetzki, S, Golan, I, Melnik, L, Faitelson, Y. CD44 in cancer. Crit Rev Clin Lab Sci. 2002, 39: 527-579.
29. Lokeshwar, BL, Lokeshwar, VB, Block, NL. Expression of CD44 in prostate cancer cells: association with cell proliferation and invasive potential. Anticancer Res. 1995, 15: 1191-
30. Welsh, CF, Zhu, D, Bourguignon, LY. Interaction of CD44 variant isoforms with hyaluronic acid and the cytoskeleton in human prostate cancer cells. J Cell Physiol. 1995, 64: 605-612.
31. Iczkowski, KA, Bai, S, Pantazis, CG. Prostate cancer overexpresses CD44 variants 7-9 at the messenger RNA and protein level. Anticancer Res. 2003 234: 3129-3140.
32. Gao, AC., Lou, W., Sleeman, JP, Isaacs, JT. Metastasis suppression by the standard CD44 isoform does not require the binding of prostate cancer cells to hyaluronate. Cancer Res. 1998, 58: 2350-2352.
33. Ekici, S, Ayhan, A, Kendi, S, Ozen, H. Determination of prognosis in patients with prostate cancer treated with radical prostatectomy: prognostic value of CD44v6 score. J Urol, 167: 2037-2041, 2002.
34. Aaltomaa, S, Lipponen, P, Ala-Opas, M. Kosma, VM. Expression and prognostic value of CD44 standard and variant v3 and v6 isoforms in prostate cancer. Eur Urol., 2001 39:138-144.
35.Folkman, J, Browder, T, Palmblad, J. Angiogenesis research: guidelines for translation to clinical application. Thromb Haemost. 2001, 86: 23-33.
36. Sauer, G, Deissler, H. Angiogenesis: prognostic and therapeutic implications in gynecologic and breast malignancies. Curr Opin Obstet Gynecol. 2003, 15: 45-49.
37. Goddard, JC, Sutton, CD, Furness, PN, O'Byrne, KJ, Kockelbergh, R.C. Microvessel density at presentation predicts subsequent muscle invasion in superficial bladder cancer. Clin Cancer Res. 2003, 9: 2583-2586.
38. Poon, RT, Fan, ST, Wong, J. Clinical significance of angiogenesis in gastrointestinal cancers: a target for novel prognostic and therapeutic approaches. Ann Surg. 2003, 238: 9-28.
39. Zhang, X, Yamashita, M, Uetsuki, H, Kakehi, Y. Angiogenesis in renal cell carcinoma: Evaluation of microvessel density, vascular endothelial growth factor and matrix metalloproteinases. Int J Urol. 2002, 9: 509-514.
40. De La Taille, A, Katz, AE, Bagiella, E, Buttyan, R, Sharir, S, Olsson, CA, Burchardt, T, Ennis, RD, Rubin, MA. Microvessel density as a predictor of PSA recurrence after radical prostatectomy : A comparison of CD34 and CD31. Am J Clin Pathol 2000, 113: 555-562.
41. Bono, AV, Celato, N., Cova, V., Salvadore, M., Chinetti, S., Novario, R. Microvessel density in prostate carcinoma. Prostate Cancer Prostatic Dis. 2002, 5: 123-127.
42. Weidner, N., Carroll, P.R., Flax, J., Blumenfeld, W., Folkman, J. Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. Am J Pathol 1993, 143: 401-409.
43. Silberman, M.A., Partin, A.W., Veltri, R.W., Epstein, J.I. Tumor angiogenesis correlates with progression after radical prostatectomy but not with pathologic stage in Gleason sum 5 to 7 adenocarcinoma of the prostate. Cancer 1997, 79: 772-779.
44. Gettman, M.T., Bergstralh, E.J., Blute, M., Zincke, H., Bostwick, D.G. Prediction of patient outcome in pathologic stage T2 adenocarcinoma of the prostate: Lack of significance for microvessel density analysis. Urology 1998, 51:79-85.
45. Rubin, M.A., Buyyounouski, M., Bagiella, E., Sharir, S., Neugut, A., Benson, M., De La Taille, A., Katz, A.E., Olsson, C.A., Ennis, R.D. Microvessel density in prostate cancer : Lack of correlation with tumor grade, pathologic stage, and clinical outcome. Urology 1999, 53: 542-547.
46. Ekici S., Cerwinka, W.H., Duncan, R. C. Gomez, P., Civantos, F., Soloway, M.S., Lokeshwar, V.B. Comparison of the prognostic potential of hyaluronic acid, hyaluronidase (HYALI-1), CD44v6 and Microvessel density for prostate cancer (Submitted to Int. J. Cancer, 2004).
47. Lokeshwar, V.B., Schroeder, G.L., Carey, R.I., Soloway, M.S., Iida, N. Regulation of hyaluronidase activity by alternative mRNA splicing. J Biol Chem. 277: 33654-33663, 2002.

48. Lokeshwar, V.B., Young M.J., Goudarzi, G., Iida, N., Yudin, A.I., Cherr, G.N., Selzer, M.G. Identification of bladder tumor-derived hyaluronidase: Its similarity to HYAL1. Cancer Res., 59: 4464-4470, 1999.
49. Patel, S., Turner, P.R., Stubberfield, C., Barry, E., Rohlff, C.R., Stamps, A., McKenzie, E., Young, K., Tyson, K., Terrett, J., Box, G., Eccles, S., Page, M.J. Hyaluronidase gene profiling and role of hyal-1 overexpression in an orthotopic model of prostate cancer. Int J Cancer 97: 416-24. Erratum 98: 957, 2002.
50. Imamura, H., Takao, S., Aikou, T. A modified invasion –3-(4,5, Dimethylthiazole-2-yl)-2,5, diphenyl-tetrazolium bromide assay for quantitating tumor cell invasion. Cancer Research 54: 3620-3624, 1994.
51. Church, D., Zhang, Y., Rago, R. Efficacy of suramin against human prostate carcinoma DU145 xenografts in nude mice. Cancer Chemother Pharmacol., 43: 198-204, 1999.

[CANCER RESEARCH 63, 2638–2644, May 15, 2003]

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

J. Timothy Posey, Mark S. Soloway, Sinan Ekici, Mario Sofer, Francisco Civantos, Robert C. Duncan, and Vinata B. Lokeshwar[2]

Departments of Urology [J. T. P., M. S. S., S. E., M. S., F. C., V. B. L.], Department of Epidemiology [R. C. D.], and Cell Biology and Anatomy [V. B. L.], University of Miami School of Medicine, Miami, Florida 33101

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-

Received 11/2/02; accepted 3/12/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by Department of Defense Grant DAMD 170210005, NCI Grant RO1 CA 72821, and Sylvester Comprehensive Cancer Center and Department of Urology, University of Miami.

[2] To whom requests for reprints should be addressed, at Department of Urology (M-800), University of Miami School of Medicine, P.O. Box 016960, Miami, FL, 33101. Phone: (305) 243-6321; Fax: (305) 243-6893; E-mail: vlokeshw@med.Miami.edu.

[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,

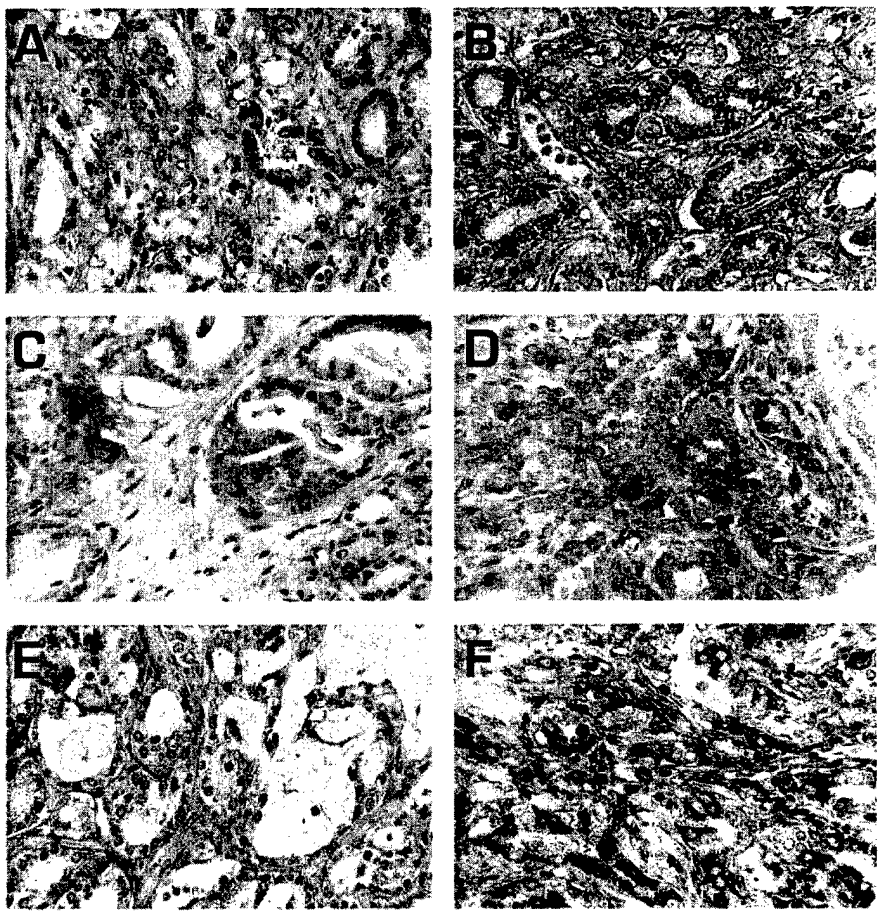


Fig. 1. HA staining of CaP specimens from nonprogressed and progressed patients. HA was localized in CaP tissues using a biotinylated HA-binding protein and a streptavidin peroxidase DAB-chromogen detection system. *A*, *C*, and *E*, HA staining in specimens from nonprogressed patients. *B*, *D*, and *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

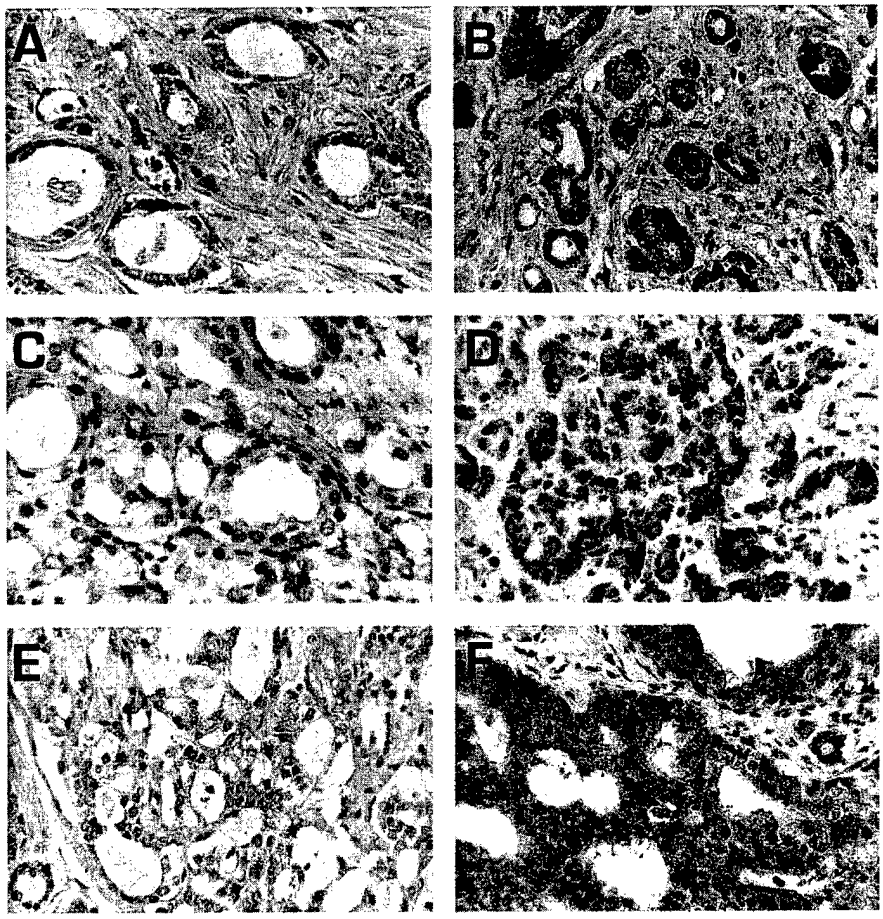


Fig. 3. HYAL1 staining of CaP specimens from nonprogressed and progressed patients. HYAL1 was localized in CaP tissues using an anti-HYAL1 antibody and a streptavidin peroxidase DAB-chromogen detection system. *A*, *C*, and *E*, HYAL1 staining in specimens from nonprogressed patients. *B*, *D*, and *F*, 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.

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.

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 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.

ACKNOWLEDGMENTS

We thank Dr. Anil Vaidya for helping in gathering some of the follow-up information. We thank Pamela Roza for her help with the illustrations.

REFERENCES

1. Etzioni, R., Penson, D. F., Legler, J. M., di Tommaso, D., Boer, R., Gann, P. H., and Feuer, E. J. Overdiagnosis due to prostate-specific antigen screening: lessons from U.S. prostate cancer incidence trends. J. Natl. Cancer Inst., *94:* 981–990, 2002.
2. Yao, S. L., and Lu-Yao, G. Understanding and appreciating overdiagnosis in the PSA era. J. Natl. Cancer Inst., *94:* 958–960, 2002.
3. Muzzonigro, G., and Galosi, A. B. Biological selection criteria for radical prostatectomy. Ann. NY Acad. Sci., *963:* 204–212, 2002.
4. Small, E. J., and Roach M., III. Prostate-specific antigen in prostate cancer: a case study in the development of a tumor marker to monitor recurrence and assess response. Semin. Oncol., *29:* 264–273, 2002.
5. Palisaan, R. J., Graefen, M., Karakiewicz, P. I., Hammerer, P. G., Huland, E., Haese, A., Fernandez, S., Erbersdobler, A., Henke, R. P., and Huland, H. Assessment of clinical and pathologic characteristics predisposing to disease recurrence following radical prostatectomy in men with pathologically organ-confined prostate cancer. Eur. Urol., *41:* 155–161, 2002.
6. Waltregny, D., de Leval, L., Coppens, L., Youssef, E., de Leval, J., and Csatronovo, V. Detection of the 67-kD laminin receptor in prostate cancer biopsies as a predictor of recurrence after radical prostatectomy. Eur. Urol., *40:* 495–503, 2002.
7. Pound, C. R., Partin, A. W., Epstein, J. I., and Walsh, P. C. Prostate-specific antigen after anatomic radical retropubic prostatectomy. Patterns of recurrence and cancer control. Urol. Clin. North Am., *24:* 395–406, 1997.
8. Pettaway, C. A. Prognostic markers in clinically localized prostate cancer. Tech. Urol., *4:* 35–42, 1998.
9. Blute, M. L. Bergstralh, E. J., Iocca, A., Scherer, B., and Zincke, H. Use of Gleason score, prostate specific antigen seminal vesicle and margin status to predict biochemical failure after radical prostatectomy. J. Urol., *165:* 119–125, 2001.
10. Lokeshwar, V. B., Rubinowicz, D., Schroeder, G. L., Forgacs, E., Minna, J. D., Block, N. L. Nadji. M., and Lokeshwar, B. L. Stromal and epithelial expression of tumor markers hyaluronic acid and hyaluronidase in prostate cancer. J. Biol. Chem., *276:* 11922–11932, 2001.
11. Lokeshwar, V. B., Lokeshwar. B. L., Pham, H. T., and Block, N. L. Association of hyaluronidase, a matrix-degrading enzyme, with prostate cancer progression. Cancer Res., *56:* 651–657, 1996.
12. Tammi, M. I., Day, A. J., and Turley, E. A. Hyaluronan and homeostasis: a balancing act. J. Biol. Chem., *277:* 4581–4584, 2002.
13. Tool, B. P. Hyaluronan is not just a goo! J. Clin. Investig., *106:* 335–336, 2000.
14. Delpech, B., Girard, N., Bertrand, P., Courel, N. M., Chauzy, C., and Delpech, A. Hyaluronan: fundamental principles and applications in cancer. J. Intern. Med., *242:* 41–48, 1997.
15. Turley EA, Noble PW, and Bourguignon LY. Signaling properties of hyaluronan receptors. J. Biol. Chem., *277:* 4589–4592, 2002.
16. Hautmann, S. H., Lokeshwar, V. B., Schroeder, G. L., Civantos, F., Duncan, R. C., Friedrich, M. G., and Soloway, M. S. Elevated tissue expression of hyaluronic acid and hyaluronidase validate HA-HAase urine test for bladder cancer. J. Urol., *165:* 2068–2074, 2000.
17. Setala, L. P., Tammi, M. I., Tammi, R. H., Eskelin, M. J., Lipponen, P. R., Argen, U. M., Parkkinen, J., Alhava, E. M., and Kosma, V. M. Hyaluronan expression in gastric cancer cells is associated with local and nodal spread and reduced survival rate. Br. J. Cancer, *79:* 1133–1138, 1999.
18. Auvinen, P., Tammi, R., Parkkinen, J., Tammi, M., Agren, U., Johansson, R., Hirvikoski, P., Eskelinen, M., and Kosma, V. M. Hyaluronan in peritumoral stroma and malignant cells associates with breast cancer spreading and predicts survival. Am. J. Pathol., *156:* 529–536, 2000.
19. Pirinen, R., Tammi, R., Tammi, M., Hirvikoski, P., Parkkinen, J. J., and Johansson, R. Prognostic value of hyaluronan expression in non-small-lung cancer: increased stromal expression indicates unfavorable outcome in patients with adenocarcinoma. Int. J. Cancer, *95:* 12–27, 2001.
20. Knudson, W. Tumor associated hyaluronan: providing an extracellular matrix that facilitates invasion. Am. J. Pathol., *148:* 1721–1726, 1996.

21. Ropponen, K., Tammi, M., Parkkinen, J., Eskelinen, M., Tammi, R., Lipponen, P., Agren, V., Alhava, E., and Kosma, V. M. Tumor-associated hyaluronan as an unfavorable prognostic factor in colorectal cancer. Cancer Res., *58:* 342–347, 1998.
22. Lipponen, P., Aaltomaa, S., Tammi, R., Tammi, M., Agren, U., and Kosma, V. M. High stromal hyaluronan level is associated with poor differentiation and metastasis in prostate cancer. Eur. J. Cancer, *37:* 849–856, 2001.
23. Lokeshwar, V. B., Obek, C., Pham, H. T., Wei, D. C., Young, M. J., Duncan, R. C., Soloway, M. S., and Block, N. L. Urinary hyaluronic acid and hyaluronidase: markers for bladder cancer detection and evaluation of grade. J. Urol., *163:* 348–356, 2000.
24. Lokeshwar, V. B., Obek, C., Soloway, M. S., and Block, N. L. Tumor-associated hyaluronic acid: a new sensitive and specific urine marker for bladder cancer. Cancer Res., *57:* 773–777, 1997.
25. Liu, N., Lapcevich, R. K., Underhill, C. B., Han, Z., Gao, F., Swartz, G., Plum, S. M., Zhang, L., and Grec, S. J. Metastatin: a hyaluronan-binding complex from cartilage that inhibits tumor growth. Cancer Res., *61:* 1022–1028, 2001.
26. Hayen, W., Goebeler, M., Kumar, S., Riessen, R., and Nehls, V. Hyaluronan stimulates tumor cell migration by modulating the fibrin fiber architecture. J. Cell Sci., *112:* 2241–2251, 1999.
27. Hoborth, K., Maier, U., and Marberger, M. Topical chemoprophylaxis of superficial bladder cancer by mitomycin C and adjuvant hyaluronidase. Eur. Urol., *21:* 206–210, 1992.
28. Itano, N., Atsumi, F., Sawai, T., Yamada, Y., Miyaishi, O., Senga, T., Hamguchi, M., and Kimata, K. Abnormal accumulation of hyaluronan matrix diminishes contact inhibition of cell growth and promotes cell migration. Proc. Natl. Acad. Sci. USA, *99:* 3609–3614, 2002.
29. Roden, L., Campbell, P., Fraser, J. R. E., Laurent, T. C., Petroff, H., and Thompson, J. N. Enzymatic pathways of hyaluronan catabolism. In: E. Whelan (ed.), The Biology of Hyaluronan. Ciba Foundation Symp. No. 143, pp. 60–86. Chichester, New York: Wiley, 1989.
30. Jedrzejas, M. J. Structural and functional comparison of polysaccharide-degrading enzymes. Crit. Rev. Biochem. Mol. Biol., *35:* 221–251, 2000.
31. West, D. C., Hampson, I. N., Arnold, F., and Kumar, S. Angiogenesis induced by degradation products of hyaluronic acid. Science (Wash. DC), *228:* 1324–1326, 1985.
32. Lokeshwar, V. B., and Selzer, M. G. Differences in hyaluronic acid-mediated functions and signaling in arterial, microvessel, and vein-derived human endothelial cells. J. Biol. Chem., *275:* 27641–27649, 2000.
33. Csoka, A. B., Frost, G. I., and Stern, R. The six hyaluronidase-like genes in the human and mouse genomes. Matrix Biol., *20:* 499–508, 2001.
34. Lepperdinger, G., Mullegger, J., and Kreil, G. Hyal2–less active, but more versatile? Matrix Biol., *20:* 509–514, 2001.
35. Cherr, G. N., Yudin, A. I., and Overstreet, J. W. The dual functions of GPI-anchored PH-20: hyaluronidase and intracellular signaling. Matrix Biol., *20:* 515–525, 2001.
36. Lokeshwar, V. B., Soloway, M. S., and Block, N. L. Secretion of bladder tumor-derived hyaluronidase activity by invasive bladder cancer cells. Cancer Lett., *131:* 21–27, 1998.
37. Lokeshwar, V. B., Young, M. J., Goudarzi, G., Iida, N., Yudin, A. I., Cherr, G. N., and Selzer, M. G. Identification of bladder tumor-derived hyaluronidase: its similarity to HYAL1. Cancer Res., *59:* 4464–4470, 1999.
38. Godin, D. A., Fitzpatrick, P. C., Scandurro, A. B., Belafsky, P. C., Woodworth, B. A., Amedee, R. G., Beech, D. J., and Beckman, B. S. PH20: a novel tumor marker for laryngeal cancer. Arch. Otolaryngol. Head Neck Surg., *126:* 402–404, 2000.
39. Beech, D. J., Madan, A. K., and Deng, N. Expression of PH-20 in normal and neoplastic breast tissue. J. Surg. Res., *103:* 203–207, 2002.
40. Lokeshwar, V. B., Schroeder, G. L., Carey, R. I. Soloway, M. S., and Iida, N. Regulation of hyaluronidase activity by alternative mRNA splicing. J. Biol. Chem., *277:* 33654–33663, 2002.
41. Sweat, S. D., Bergstralh, E. J., Slezak, J., Blute, M. L., and Zincke, H. Competing risk analysis after radical prostatectomy for clinically nonmetastatic prostate adenocarcinoma according to clinical Gleason score and patient age. J. Urol., *168:* 525–529, 2002.
42. Fradet, Y. Role of radical prostatectomy in high-risk prostate cancer. Can. J. Urol., *9:* 8–13, 2002.
43. Klein, E. A., and Kupelian, P. A. Localized prostate cancer. Curr. Treat. Options Oncol., *1:* 433–445, 2000.
44. Isaacs, W., De Marzo, A., and Nelson, W. Focus on prostate cancer. Cancer Cell, *2:* 113–116, 2002.
45. Feroze-Merzoug, F., Schober, M. S., and Chen, Y. Q. Molecular profiling in prostate cancer. Cancer Metastasis Rev., *20:* 165–171, 2001.
46. Claudio, P. P., Zamparelli, A., Garcia, F. U., Claudio, L., Ammirati, G., Farina, A., Bovicelli, A., Russo, G., Giordano, G. G., McGinnis, D. E., Giordano, A., and Cardi, G. Expression of cell-cycle-regulated proteins pRb2/p130, p107, p27(kip1), p53, mdm-2, and Ki-67 (MIB-1) in prostatic gland adenocarcinoma. Clin. Cancer Res., *8:* 1808–1815, 2002.
47. Asmann, Y. W., Kosari, F., Wang, K., Cheville, J. C., and Vasmatzis, G. Identification of differentially expressed genes in normal and malignant prostate by electronic profiling of expressed sequence tags. Cancer Res., *62:* 3308–3314, 2002.
48. Vis, A. N., van Rhijn, B. W., Noordzij, M. A., Schroder, F. H., and van der Kwast, T. H. Value of tissue markers p27(kip1), MIB-1, and CD44s for the pre-operative prediction of tumour features in screen-detected prostate cancer. J. Pathol., *197:* 148–154, 2002.
49. Ross, J. S., Sheehan, C. E., Fisher, H. A., Kauffman, R. A., Dolen, E. M., and Kallakury, B. V. Prognostic markers in prostate cancer. Expert. Rev. Mol. Diagn., *2:* 129–142, 2002.
50. Pham, H. T., Block, N. L., and Lokeshwar, V. B. Tumor derived hyaluronidase: a diagnostic urine marker for high-grade bladder cancer. Cancer Res., *57:* 778–783, 1997.
51. Hiltunen, E. L., Anttila, M., Kultti, A., Ropponen, K., Penttinen, J., Yliskoski, M., Kuronen, A. T., Juhola, M., Tammi, R., Tammi, M., and Kosma, V. M. Elevated hyaluronan concentration without hyaluronidase activation in malignant epithelial ovarian tumors. Cancer Res., *62:* 6410–6413, 2002.
52. Jacobson, A., Rahmanian, M., Rubin, K., and Heldin, P. Expression of hyaluronan synthase 2 or hyaluronidase 1 differentially affect the growth rate of transplantable colon carcinoma cell tumors. Int. J. Cancer, *102:* 212–219, 2002.
53. Patel, S., Turner, P. R., Stubberfield, C., Barry, E., Rohlff, C. R., Stamps, A., McKenzie, E., Young, K., Tyson, K., Terrett, J., Box, G., Eccles, S., and Page, M. J. Hyaluronidase gene profiling and role of hyal-1 overexpression in an orthotopic model of prostate cancer. Int. J Cancer, *97:* 416–424, 2002.

Submitted to International Journal of Cancer January 5, 2004

COMPARISON OF THE PROGNOSTIC POTENTIAL OF HYALURONIC ACID, HYALURONIDASE (HYAL-1), CD44v6 AND MICROVESSEL DENSITY FOR PROSTATE CANCER

Sinan Ekici[1], Wolfgang H. Cerwinka[1], Robert Duncan[2], Pablo Gomez[1], Francisco Civantos[1], Mark S. Soloway[1], Vinata B. Lokeshwar[1,3,4]

Departments of Urology (1), Epidemiology (2), Sylvester Comprehensive Cancer Center (3) and Cell Biology and Anatomy (4) University of Miami School of Medicine, Miami, Florida 33101

RUNNING TITLE: Prognostic Indicators for Prostate Cancer

*: **Address for correspondence:**
Vinata B. Lokeshwar, Ph.D.
Department of Urology (M-800)
University of Miami School of Medicine
P.O. Box 016960
Miami, Florida, 33101

Phone: (305) 243-6321

Fax: (305) 243-6893

e-mail: vlokeshw@med.miami .edu

Key words: Prostate cancer, prognostic indicators, hyaluronic acid, hyaluronidase, HYAL-1, CD44v6, microvessel density

Abbreviations used: PCa: prostate cancer/cancer of the prostate; **EPE:** extra-prostatic extension; **HA:** hyaluronic acid; **HAase:** hyaluronidase; **IHC:** immunohistochemistry /immunohistochemical; **MVD:** microvessel density; **PSA:** Prostate specific antigen. **ROC:** receiver-operating characteristic

ABSTRACT

Purpose: Despite the development of nomograms designed to evaluate a prostate cancer (PCa) patient's prognosis, the information has been limited to prostate specific antigen (PSA), clinical stage, Gleason score and tumor volume estimates. We compared the prognostic potential of 4 histological markers, hyaluronic acid (HA), HYAL-1 type hyaluronidase (HAase), CD44v6 and microvessel density using immunohistochemistry. HA is a glycosaminoglycan that promotes tumor metastasis. CD44 glycoproteins serve as cell surface receptors for HA and CD44v6 isoform is associated with tumor metastasis. HYAL-1 type HAase is expressed in tumor cells and like other HAases degrades HA into angiogenic fragments.

Materials and Methods: Archival PCa specimens (n = 66) were obtained from patients who underwent radical prostatectomy for clinically localized PCa and on whom there was a minimum follow-up of 72 months (range 72 – 131 months, mean follow-up 103 months). For HA, HYAL-1 and CD44v6 staining and MVD determination, a biotinylated HA-binding protein, an anti-HYAL-1 IgG, an anti-CD44v6 IgG and an anti-CD34 IgG were used, respectively. HA and HYAL-1 staining was evaluated as low- and high-grade. CD44v6 staining and MVD were evaluated quantitatively and then grouped as low- and high-grade.

Results: At 72 month cut-off limit for evaluating biochemical recurrence, HA, HYAL-1, combined HA-HYAL-1, CD44v6 and MVD staining predicted progression with 96%, 84%, 84%, 68% and 76% sensitivity, respectively. The specificity was, 61% (HA), 80.5% (HYAL-1) and 87.8% (HA-HYAL-1), 56.1% (CD44v6) and 61% (MVD), respectively. The sensitivity and specificity values for each marker did not change significantly in a subset of 45 patients on whom > 112-month follow-up was available. In univariate analysis using Cox Proportional Hazard model, preoperative PSA, Gleason sum, margin status, seminal vesicle, extra-prostatic extension (EPE), HA, HYAL-1 and HA-HYAL-1 and MVD, but not CD44v6, age and clinical stage, were significant in predicting biochemical recurrence ($P < 0.05$). In the multivariate analysis using stepwise selection, only preoperative PSA (Harzard ratio/unit PSA change 1.086; $P < 0.0001$), EPE (Hazard ratio 6.22; $P = 0.0016$), and HYAL-1 (8.196; $P = 0.0009$)/HA-HYAL-1 (Hazard ratio, 5.191; $P = 0.0021$) were independent predictors of biochemical recurrence. HA was an independent predictor of prognosis, if HYAL-1 staining inference was not included in the multivariate model.

Conclusion: In this retrospective 72 to 131 follow-up study, EPE and preoperative PSA and HYAL-1 either alone or together with HA (i.e., combined HA-HYAL-1), were found to be independent prognostic indicators for PCa.

INTRODUCTION

Over the last decade, the number of curable prostate cancer (PCa) cases has significantly increased due to the widespread use of prostate specific antigen (PSA) (1,2). However, despite a careful selection of patients, the disease recurs in a substantial percentage of localized PCa cases undergoing curative treatment modalities (i.e., radical prostatectomy and radiotherapy) (3-6). An accurate prediction of the risk of progression would be useful in choosing the type and the timing of the most appropriate treatment. Although existing parameters such as, the Gleason sum or pre-operative PSA provide some prognostic information, it is difficult to estimate prognosis in PCa patients, since two thirds of them have PCa with Gleason sum 5 to 7 and serum PSA levels between 4 and 10 ng/ml (6-11). Furthermore, all patients with the same pathological stage and/or grade do not have the same prognosis. Thus, there is a need for accurate prognostic markers to identify the biological behavior of the tumor. Recently, we showed that HYAL-1type hyaluronidase (HAase) either alone or in combination with hyaluronic acid (HA) appears to be an independent predictor of biochemical recurrence among radical prostatectomy patients (12).

HA is a nonsulfated glycosaminoglycan made up of repeated disaccharide units, D-glucuronic acid and N-acetyl-D-glucosamine (13). HA maintains the osmotic balance of tissues and regulates cellular processes such as adhesion, migration and proliferation (13). The biological functions of HA are mediated by different HA receptors, including CD44 (14). The concentration of HA is elevated in several tumor types, and in some tumors (e.g., breast, colon, etc.), high level of HA expression in tumor-associated stroma and/or tumor cells predicts poor survival (15-19). Increased urinary HA levels serve as an accurate diagnostic marker for bladder cancer, regardless of tumor grade and stage (20). However in PCa, HA is not an independent predictor for prognosis (12,19).

HYAL-1 type HAase is present in serum and is also produced by bladder, prostate and head and neck cancer cells (21,24). HAase class of enzymes is known to degrade HA into small fragments, some of which (3-25 disaccharide units) induce angiogenesis (25,26). Angiogenic HA fragments stimulate endothelial cell proliferation, adhesion and migration by activating focal adhesion kinase and mitogen-activated protein kinase pathways (26). We have previously shown the presence of angiogenic HA fragments in PCa tissues and in the urine and saliva of bladder and head and neck cancer patients, respectively (22,24,27). In a retrospective study with a minimum of 5-year follow-up, we showed that HYAL-1 staining predicts progression with 84% sensitivity and 80% specificity (12). Furthermore, high HYAL-1 staining was an independent predictor for prognosis.

CD44 denotes a family of cell surface transmembrane glycoproteins involved in cell-to-cell and cell-to-extracellular matrix interactions (28,29). Alternative splicing of CD44 mRNA in 10 of the 20 exons generates several variant CD44 isoforms (29,30). The standard form of CD44 (i.e., CD44s) is an HA receptor and is expressed in a variety of normal and tumor cell types (28,31). We have previously shown that an isoform of CD44 (ex14/v10) is involved in HA-mediated endothelial cell proliferation (32). The correlation between tumor progression and CD44s and/or its isoforms is controversial. We and others have shown that the androgen insensitive prostate cancer line PC-3 and primary PCa cells express CD44s and CD44 variants (e.g., CD44v3 and CD44v6), however, the androgen sensitive poorly metastatic line LNCaP does not express CD44 (33-35). Contrary to these findings, it has been shown that the over-expression of CD44v6 in a rat PCa line decreases metastasis (36). Recently, Ekici et al showed that decreased expression of CD44v6 could be a predictor of poor prognosis in clinically localized PCa (37). Aaltomaa et al also reported similar results (38).

Angiogenesis is an essential process for tumor growth and metastasis (39-41). Clinical significance of angiogenesis, measured as microvessel density (MVD), has been demonstrated for several tumor types, including, gastrointestinal, breast, bladder and renal

cell carcinomas (42-45). Studies that compared various endothelial cell markers (i.e., CD31, CD34 and Factor VIII) have shown that CD34 is a sensitive endothelial cell marker for measuring MVD (46,47). At the present time, the clinical significance of MVD, as an independent predictor of pathological stage and recurrence in PCa remains controversial (46-51).

In this study, we compared the prognostic potential of markers, HA, CD44v6, HYAL-1 and MVD in regards to clinically localized PCa. Since HYAL-1 degrades HA and generates angiogenic fragments and CD44 acts as a cell-surface receptor for both HA and HA fragments, it is interesting to examine whether these biologically linked molecules are accurate prognostic indicators for PCa, and whether they influence each other's prognostic capabilities.

MATERIALS AND METHODS

Specimens and study patients:

Sixty-six randomly selected PCa specimens were obtained from patients who underwent radical retropubic prostatectomy for clinically localized PCa between 1992 and 1995 at University of Miami. The patients neither received neo-adjuvant hormonal therapy nor had metastasis to regional pelvic lymph nodes. The minimum available follow-up on all patients was 72 months. The study was conducted under a protocol approved by University of Miami's Institutional Review Board. Of the 66 patients, 25 patients had biochemical or clinical recurrence before 72 months (mean time to recurrence: 21.3 months; range: 3 to 61 months), and 41 patients were free of disease recurrence (mean follow-up: 103 months; range: 72 - 131 months). Biochemical recurrence was defined as a PSA level $\geq$ 0.4 ng/ml in 2 successive measurements after the operation, in which case the first date of elevated PSA level was considered as the date of failure. The patient characteristics with respect to age, preoperative PSA, and tumor (i.e., Gleason sum, stage, margin, extraprostatic extension (EPE) and seminal vesicle invasion) are shown in Table 1.

Immunohistochemistry and slide grading:

For all specimens, paraffin embedded blocks containing PCa tissues representing the major Gleason score were selected by the pathologist of the study. From each block, 8 slides were prepared. Four slides were used for HA, HYAL-1, CD44v6 and anti-CD34 (for MVD determination) staining. The remaining slides were either used for determining non-specific staining corresponding to each staining reagent or for repeating the staining to evaluate staining consistency. For all staining procedures, the specimen slides were deparaffinized, rehydrated and treated with an Antigen Retrieval Solution (Dako Laboratories).

HA and HYAL-1 staining: IHC for localizing HA and HYAL-1 in PCa tissues was carried out as described previously (12, 22). HA was localized in PCa tissues using a biotinylated HA-binding protein. HYAL-1 was localized using a rabbit polyclonal anti-HYAL-1 IgG, which was generated against a peptide sequence present in the HYAL-1 protein (amino acids 321 to 338) (12,22).

Two readers 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 HYAL-1 was graded as 0 (no staining), 1+, 2+, and 3+. For HA staining, both the tumor-associated stroma and tumor cells were graded in each slide. 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+ and 2+ staining, and high-grade staining included 3+ intensity. 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 HYAL-1, high-grade staining represented 2+ and 3+ staining, whereas low-grade staining included 0 and 1+ staining intensities. For the combined HA-HYAL-1 staining, a positive result was indicated only when both HA (stromal, tumor cells, or both) and HYAL-1 staining intensities were of high grade. Any other combination was considered negative. All slides were reviewed out of order to prevent direct comparison of individual cases for HA and HYAL-1.

CD34 staining: Following the antigen retrieval step (as described above), the slides were incubated with a mouse monoclonal anti-human hematopoietic progenitor cell CD34 antibody (dilution of 1:20; DAKO, Denmark) at 4°C for 15 hours. The slides were then incubated with a biotinylated anti-mouse antibody and an avidin-peroxidase conjugate solution (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA). To visualize peroxidase binding sites, the slides were incubated with a 3,3'-diaminobenzidine (DAB) chromogen substrate solution (Dako Laboratories) for 10 minutes. The slides were counterstained with hematoxylin, dehydrated and mounted.

The method described by Weidner et al (48) was used for scoring of the microvessels stained with CD34. The area of the highest MVD in each tissue specimen was localized under 40X magnification and was designated as "hot spot". The microvessels in the hot spots were counted under 400X-magnification. Any vessel with lumen and endothelial cell or endothelial cell cluster stained positively for CD34 was considered to be a single countable microvessel. MVD count was defined as the mean value of the counts obtained in 3 separate, contiguous but not overlapping areas within the hot spot. A cutoff value was determined using the receiver operating characteristic (ROC) curve and according to this value two groups of low and high MVD were assigned. Microvessels were examined and counted by the three readers (S.E., V.B.L. and W.H.C.) independently and without the knowledge of the clinical and pathological status of the patients. The sections were reviewed out of order to prevent direct comparison of individual cases for CD34.

CD44v6 staining: Following antigen retrieval, the slides were incubated with a mouse monoclonal anti-human CD44v6 antibody (dilution of 1:50; Bender Med systems, Vienna, Austria) at 4°C for 15 hours. The sections were then incubated with a biotinylated secondary antibody and an avidin-peroxidase conjugate solution (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA). To visualize peroxidase binding sites, the slides were incubated with a 3,3'-diaminobenzidine (DAB) chromogen substrate solution (Dako Laboratories) for 10 minutes. The slides were counterstained with hematoxylin, dehydrated and mounted.

The slides for CD44v6 were scored as described by Ekici et al (37). All sections included normal prostate tissue and/or benign prostatic hyperplasia glands as internal controls. Intensity of staining was graded as 0 for no staining, 1 for weak intensity, 2 for moderate intensity and 3 for strong intensity. A combined staining score based on an estimate of the percentage of tumor cells stained and the intensity of staining was developed. The areas of tumor cells stained with maximum intensity (primary area) and the other tumor cells stained with lesser intensity (secondary area) were determined in percentage values. The combined score is obtained by adding the scores of the primary and secondary areas. Staining intensities were examined and scored by two readers (S.E. and V.B.L.), independently and in a blinded fashion. A cutoff value was determined from the ROC curve and according to this value two groups of low and high CD44v6 staining were assigned.

Statistical Analysis:

The inter-assay variability regarding staining intensity was determined by Pearson's correlation analysis. The Spearman's bivariate correlation coefficients were 0.85, 0.9, 0.98 and 0.95 for HA, HYAL-1, CD34 and CD44v6 staining, respectively. For all the markers, a high-grade staining was considered as a true positive if the patient had biochemical

recurrence. Consequently, low-grade staining was considered as a true negative, if the patient had no biochemical recurrence. The sensitivity, specificity, accuracy, positive predictive value (PPV), and negative predictive value (NPV) for HA, HYAL-1, HA-HYAL-1, CD34 and CD44v6 staining inferences were calculated using the 2 x 2 contingency table (high-grade/low-grade staining and progressed/non-progressed CaP patients) at 72, 84, 100 and 112 month cut-off limits. For CD44v6 and MVD, Receiver Operating Characteristic curves were developed for determining the optimal cut-off limits that yielded the best possible sensitivity and specificity values. The cut-off limits for CD44v6 and MVD were 180 and 41, respectively. The sensitivity is defined as: true positive (i.e., No. of recurred patients predicted by a marker) ÷ total no. of recurred patients. Specificity: true negatives (i.e., No. of non-recurred patients predicted by a marker) ÷ total no. of non-recurred patients. Accuracy: No. of True positive + No. of true negatives ÷ total no. of CaP patients in the study. PPV: No. of true positive ÷ No. of true positive + No. of false positive. NPV: No. of true negative ÷ No. of true negative + No. of false negative. The data on various, biochemical, surgical, and pathologic parameters, as well as HA, HYAL-1, HA-HYAL-1, CD34 and CD44v6 staining inferences, were analyzed by Cox Proportional Hazard model, using single variable analysis (univariate analysis) or step-wise selection analysis. Stratified Kaplan-Meier analyses were performed on the variables that were found to be significant in the multivariate Cox Proportional Hazard model. For PSA subset analysis, Mantael-Haenszel Chi-square analysis or Student's t test were used to determine statistical significance. Statistical analysis was carried out using the SAS Software Program (version 8.02; SAS Institute, Cary, NC).

RESULTS:

Immunohistochemistry of the tissue markers:

The HA, HYAL-1, CD44v6 and CD34 antigens were localized in 66 archival PCa specimens obtained from patients who underwent radical retropubic prostatectomy for clinically localized disease. An increase in PSA levels ≥ 0.4 ng/ml was taken as an indicator of biochemical recurrence. Fig. 1 shows immunohistochemical localization of HA, HYAL-1, CD44v6 and MVD in 2 Gleason 7 PCa specimens, one each from a non-recurred (panels A, C, E, G) and a recurred (panels B, D, F, H) patient, respectively.

As shown in Fig. 1 panel A, very little HA staining is seen in PCa tissue from a patient who did not progress within 72 months. Among the 41 PCa specimens from non-recurred patients, 25 showed low-grade staining. Panel B shows high-grade HA staining in PCa specimen from a patient who had biochemical recurrence in < 72 months (median time to recurrence: 19 months; mean time to recurrence: 21.3 months). The HA staining is seen mainly in tumor-associated stroma. However, high-grade HA staining was also seen in tumor cells in 8 out of 25 patients who had biochemical recurrence. Out of the 25 patients who had recurred, 24 showed high-grade HA staining.

An anti-HYAL-1 peptide IgG was used to localize HYAL-1. As shown in Fig. 1 panel C, little HYAL-1 staining is seen in the PCa tissue from a non-recurred patient. Out of the 41 non-recurred patients, PCa specimens from 33 had low-grade staining. In the PCa specimen from a patient who later recurred, high-grade HYAL-1 staining is seen (Fig. 1, panel D). The HYAL-1 expression is seen exclusively in tumor cells. Out of the 25 patients who recurred within 72 months, 21 had high-grade HYAL-1 staining.

CD44v6 was localized using an anti-CD44v6 mouse monoclonal antibody. Contrary to some earlier reports (37,38), low-grade CD44v6 staining is observed in the PCa specimen from a non-recurred patient (Fig. 1, panel E) and high-grade CD44v6 staining is observed in the PCa tissue from a recurred patient (Fig. 1, panel F). CD44v6 staining is mostly associated with the plasma membrane of tumor cells. We also observed CD44v6 in non-neoplastic epithelial cells in normal prostate and benign prostatic hyperplasia glands. However, the staining intensity of CD44v6 in non-neoplastic cells was less than that in tumor

cells. It is noteworthy that there was a great degree of heterogeneity in CD44v6 staining. For these reasons we used a semi-quantitative method to grade CD44v6 staining (37). Using a cut-off limit of 180 on the scoring scale, 23 out of 41 PCa specimens from non-recurred patients showed low-grade staining, whereas, out of the 25 patients who recurred, 17 showed high-grade staining.

It has been shown previously that visualization and scoring of microvessels using anti-CD34 staining is both sensitive and specific (46,47,52). We therefore used an anti-CD34 monoclonal antibody to visualize microvessels in PCa tissues. As shown in Fig. 1 panel G, MVD is low in the PCa tissue from a non-recurred patient. As determined from the Receiver Operating Characteristic curve, a cut-off limit of 41 was set to score low or high MVD. Out of the 41 non-recurred patients, PCa tissues from 25 patients had low MVD. However, the MVD was high in 19 out of 25 PCa tissues obtained form patients who had a recurrence. Fig. 1 panel H shows high MVD in the PCa specimen from a patient who later recurred.

Determination of sensitivity, specificity, accuracy, PPV and NPV: In this study on all of the patients a minimum 72-month follow-up was available (Mean follow-up, 103 months; median follow-up 104.2 months; range 72 – 131 months). Therefore, we determined sensitivity, specificity, accuracy, PPV and NPV of HA, HYAL-1, combined HA-HYAL-1, CD44v6, and MVD at 72-, 84-, 100- and 112-months of follow-up. As shown in Tables 2 A and 2 B, at 72 months the sensitivity of HA, HYAL-1, combined HA-HYAL-1, CD44v6, and MVD for predicting PCa recurrence is 96%, 84%, 84%, 76% and 68%, respectively. The specificity, of HA (61%), CD44v6 (56.1%), and MVD (61%) was lower than that of HYAL-1 (80.5%) and combined HA-HYAL-1 (87.8%). The accuracy of the HA-HYAL-1 (86.4%) was the highest, followed by HYAL-1 (81.8%), HA (74.2%), MVD (66.7%), and CD44v6 (57.6%). Due to higher specificity, the PPV of combined HA-HYAL-1 (80.8%) and HYAL-1 (70%) was high. However, the PPV of the CD44v6 was the lowest (48.6%), followed by MVD (54.3%) and HA (60%) staining. Due to high sensitivity, the NPV of the HA staining (96.1%) was the highest, followed by HA-HYAL-1 (90%), HYAL-1 (89.3%), MVD (80.6%) and CD44v6 (74.2%), respectively (Tables 2 A and 2 B).

At 84 month follow-up, the cohort consisted of 61 patients (Mean follow-up 107.9 months, median follow-up, 112 months; range: 85 – 131 months), out of which 36 were in the non-recurred group. Thus, the sensitivity values of all the markers remained unchanged at 84 months when compared to those at 72-month follow-up (Tables 2 A and B). There was also no significant change in the specificity, accuracy, PPV and NPV values for HA (61.1%, 75.4%, 63.2%, 95.7%), HYAL-1 (80.6%, 82%, 75%, 87.9%), combined HA-HYAL-1 (88.9%, 86.9%, 84%, 88.9%), MVD (61.1%, 67.2%, 57.6%, 78.6%), and CD44v6 (52.8%, 59%, 50% and 70.4%), respectively.

At 100-month cut-off limit, follow-up information was available on 52 patients (mean follow-up 117.1 months; median follow-up, 117.8; range 101.6 – 131). Out of these 52 patients, one who was in the non-recurred category up to 84-month follow-up showed biochemical recurrence. Interestingly, this patient was scored as a false positive on HYAL-1 and CD34 at 72- and 84-month follow-up. Thus, the sensitivity of both HYAL-1 (84.6%) and CD34 (76.9%) increased slightly, whereas that of HA (92.3%), combined HA-HYAL-1 (80.8%) and CD44v6 (65.4%) decreased (Tables 2 A and 2 B).

Follow-up information of ≥112 month was available on 45 patients (mean follow-up 121 months; median 120.2; range 112 – 131 months). At 112 month, one patient who was a false positive on HA, HYAL-1, combined HA-HYAL-1 and MVD markers up to 100 months, showed biochemical recurrence. Thus, the sensitivity of HA, HYAL-1, combined HA-HYAL-1, MVD and CD44v6 was 92.6%, 85.2%, 81.5%, 77.8% and 62.9% respectively. At final analysis, both HYAL1 and combined HA-HYAL-1 had the best specificity (94.4%, 94.4%), accuracy (88.9%, 86.7%), PPV (95.8%, 95.7%), and NPV (81%, 77.3%) values, followed by

HA (80.6%, 91.1%, 92.6%, 80.6%), MVD (77.8%, 77.8%, 84%, 70%) and CD44v6 (61.1%, 62.2%, 70.8%, 52.4%).

Evaluation of the prognostic capabilities of pre-operative and post-operative parameters and histological markers:

Univariate analysis:

Since the study patients in this cohort had variable follow-up between 72 and 131 months, we used Cox Proportional Hazard model and single parameter analysis to determine the prognostic significance of each of the pre-operative (i.e., age, PSA and clinical stage) and post-operative parameters (i.e., Gleason sum, margin +/-, EPE +/-, seminal vesicle invasion +/-), as well as, staining inferences of HA, HYAL-1, combined HA-HYAL-1, CD44v6 and MVD. As shown in Table 3, age ($p = 0.5104$; hazard ratio = 1.019), clinical stage ($p = 0.2683$, hazard ratio = 1.2620) and CD44v6 staining ($p = 0.131$ hazard ratio = 1.826) were not significant in predicting biochemical recurrence. However, pre-operative PSA ($p = 0.0006$, hazard ratio/unit PSA change = 1.048), Gleason sum overall ($p = 0.0002$; hazard ratio = 2.5), margin status ($p = 0.0003$; hazard ratio = 4.5), EPE ($p<0.0001$; hazard ratio = 12.781), seminal vesicle invasion ($p<0.0001$; hazard ratio = 6.56), HA staining ($p=0.0008$; hazard ratio = 12.091), HYAL-1 staining ($p<0.0001$; hazard ratio = 13.192), HA-HYAL-1 staining ($p<0.0001$; hazard ratio = 10.749) and MVD ($p = 0.0015$; hazard ratio = 4.36) significantly predicted biochemical recurrence (Table 3). Patients with Gleason sum $\geq$ 7 have been shown to have a greater risk of progression (41). In the single parameter analysis, the hazard of developing biochemical recurrence Gleason sum ≥ 7 patients ($p = 0.0165$, hazard ratio = 5.827), increased 2.3-fold when all Gleason sums were analyzed together (Table 3).

Multivariate analysis:

To determine the smallest number of variables that could jointly predict biochemical recurrence in this cohort of study patients, we used Cox Proportional Hazard model and step-wise selection analysis. When age, pre-operative PSA, clinical stage, Gleason sum (overall or ≥ 7), EPE, seminal vesicle invasion and staining inferences of HA, HYAL-1, CD44v6, and MVD were included in the model, only pre-operative PSA ($p < 0.0001$, hazard ratio/unit PSA change = 1.086), EPE ($p = 0.0016$, hazard ratio = 6.222) and HYAL-1 ($p = 0.0009$, hazard ratio = 8.1896) reached statistical significance in predicting biochemical recurrence (Table 4 A).

To demonstrate the joint effect of HYAL-1 and EPE or HYAL-1 and PSA on biochemical recurrence, we performed Kaplan-Meier analysis. As shown in Fig 2 A, the probability of biochemical recurrence is the highest when HYAL-1 is high and EPE is positive, and a patient has the lowest probability of recurrence when HYAL-1 is low and EPE is negative. Since PSA was a continuous estimate, with values ranging from 0.5 ng/ml to 62 ng/ml, for the entire cohort of study individuals (n = 66), we divided the cohort as those with PSA levels < 7 ng/ml and those with > 7 ng/ml. The 7 ng/ml PSA was used as the cut-off limit since that was the median PSA value for the entire cohort. As shown in Fig. 2 B, individuals with HYAL-1 high and PSA > 7 ng/ml have the highest probability of recurrence, followed by those with HYAL-1 high and PSA < 7 ng/ml. Individuals with low HYAL-1 staining and PSA levels < 7 ng/ml have the lowest probability of recurrence. These data explain why the multivariate analysis selected HYAL-1, EPE and PSA as independent prognostic indicators.

The inclusion of the combined HA-HYAL-1 staining inference instead of HA and HYAL-1 staining inferences, in the multiple regression model again showed that pre-operative PSA ($p = 0.0002$, hazard ratio/unit PSA change 1.077), EPE ($p = 0.0009$, hazard ratio = 6.906) and HA-HYAL-1 ($p = 0.0021$, hazard ratio = 5.191) were significant in

predicting biochemical recurrence (Table 4 B). None of the other pre-operative (PSA, clinical stage) and post-operative parameters (Gleason sum overall or Gleason stratification ≥ and ≤ 7, and seminal veicle) or CD44v6 and MVD staining inferences reached statistical significance in the multivariate model ($P > 0.05$, in each case). Kaplan-Meier analysis using HA-HYAL-1 and EPE or HA-HYAL-1 and PSA demonstrated that individuals with high HA-HYAL-1 and positive EPE or high HA-HYAL-1 and PSA > 7 ng/ml have the highest probability of biochemical recurrence (data not shown).

When HYAL-1 was omitted in the model during step-wise analysis, HA ($p = 0.0065$, hazard ratio = 8.658) together with pre-operative PSA ($p = 0.0006$, hazard ratio/unit PSA change = 1.079) and EPE ($p < 0.0001$, hazard ratio = 9.073) were significant in predicting biochemical recurrence. Similarly when PSA was omitted in the model, margin status reached independent prognostic significance together with EPE and HYAL-1 or HA-HYAL-1 (data not shown).

PSA sub-group analysis:

It has been suggested that biochemical recurrence < 24 months indicates systemic disease, whereas, biochemical recurrence > 24 months suggests local recurrence. To test whether any of the pre- and post-operative parameters, as well as, IHC markers under study distinguish between these groups, we performed Mantel-Haenszel chi square analysis (for testing Gleason sum, Gleason sum ≥7, EPE, margin status, seminal vesicle invasion, HA, HYAL-1, combined HA-HYAL-1 CD34 and CD44v6) or student's t test (for age and pre-operative PSA). As shown in Table 5, margin status could distinguish between PSA recurrence < 24 months and > 24 months ($P = 0.269$, $\chi^2 = 4.894$), however, none of the other parameters or markers reached statistical significance in this comparison.

DISCUSSION:

In this study we compared the prognostic potential of histologic markers HA, HYAL-1, CD44v6 and MVD for predicting biochemical recurrence in PCa patients. We chose to compare these markers since their biological functions are interrelated. For example, HA, an extracellular matrix component is a high affinity ligand for CD44 (14,28). HA-CD44 interaction promotes cell-adhesion, migration and proliferation (14,28). HYAL-1 degrades HA into small fragments, which promote angiogenesis and MVD is an indicator of angiogenesis (25,26,41). In addition to their biological relatedness, each of these histologic markers have shown potential to predict prognosis for PCa patients (12,19,37,38,46-51).

The prognostic capability of HA staining in tumors varies depending upon the tissue from where the cancer originates. HA staining in tumor-associated stroma and/or tumor cells has been shown to have prognostic capability in breast, colon and gastrointestinal cancers, however, it does not have independent prognostic capability in PCa (13-19). In this study we found that the HA staining has 92.3% sensitivity and 80.6% specificity to predict biochemical recurrence within 112 months. Interestingly, the specificity of HA staining to predict biochemical recurrence increased from 61% at 72-month follow-up, to 80.6% at 112-month follow-up. We have previously shown that at 64-month follow-up although HA staining had high sensitivity (96%), the specificity of this marker to predict biochemical recurrence was even lower (55.5%) when compared to that at 72 month (12). These results indicate that positive HA staining in PCa tissues means that the patient could have a recurrence within 112 months. In this study and in a previous study we found that HA staining shows prognostic capability in univariate analysis, however, it is not an independent predictor of biochemical recurrence. Interestingly however, if HYAL-1 staining inference is not included in the Cox Proportional Hazard model during stepwise analysis, HA together with pre-operative PSA and EPE reaches independent prognostic significance. These results indicate that HYAL-1 marker provides all of the prognostic information supplied by HA staining, as well as some additional prognostic information.

The prognostic significance of CD44 standard form and its variant isoforms (e.g., CD44v6) is unclear. Contrary to our finding that CD44v6 expression was elevated in patients who later had biochemical recurrence, two reports showed that a decrease in CD44v6 expression correlates with increase in Gleason sum and disease progression (37,38). For example, Ekici et al previously showed that CD44v6 expression inversely correlates with pathologic stage and disease progression and positively correlates with PSA-free survival (37). However in that study, CD44v6 was not an independent predictor of prognosis. Contrary to the findings of Ekici et al, Aaltomaa et al found that CD44v6 is an independent predictor of survival (38). In the present study, increased CD44v6 expression had reasonable sensitivity to predict prognosis at 72 month (68%) or 112 month (62.9%) follow-up. However, among all of the markers, it has the lowest specificity (56.1% at 72 month follow-up and 61.1% at 112-month follow-up) to predict biochemical recurrence. This low specificity may explain why CD44v6 staining is not significant in predicting biochemical recurrence in both the univariate and multivariate analyses. A likely explanation of why different studies report conflicting results regarding CD44v6 staining and prognosis for PCa is that, PCa tissues have a high degree of heterogeneity with respect to CD44v6 staining. Aaltomaa et al reported variability in CD44v6 staining intensity and in the number of tumor cells that are positive for CD44v6. Similarly Ekici et al have also reported the heterogeneous nature of CD44v6 expression and developed a semi-quantitative method for scoring CD44v6 staining (37). We used this scoring method in evaluating CD44v6 staining. Nonetheless, it is likely that the heterogeneous nature of CD44v6 will limit the prognostic significance of this marker.

Determination of MVD in PCa tissues, using anti-CD34 antibodies, has been shown to be sensitive and accurate to predict prognosis (46,47,52). Although problems exist in the methods of counting the vessels and in setting a universally accepted cut-off limit for MVD to predict recurrence, MVD has been shown to correlate with Gleason sum, pathologic stage and outcome (46-48). However, other studies have shown that MVD does not correlate with tumor grade, stage and clinical outcome (49-51). In our study, MVD at a cut-off limit of 41 showed reasonably high sensitivity both at 72-months (76%) and 112-month (77.8%) follow-up. The specificity of MVD to predict biochemical recurrence increased from 61% at 72-month follow-up to 77.8% at 112-month follow-up, suggesting that in some false positive patients, high MVD may in fact be indicative of a biochemical recurrence in < 112 months. Although in the univariate analysis MVD showed prognostic significance (Table 3), in the multivariate analysis, it had no additional prognostic significance. MVD did not reach independent prognostic significance even when HA alone, in the absence of HYAL-1 was included in the Cox model. It is possible that since HA, HYAL-1 and MVD are biologically related, all of the prognostic information provided by MVD inferences is contained in either HA or HYAL-1 staining inferences.

In this study, HYAL-1 staining alone had high sensitivity both at 72 month (84%) and ≥112-month (85.2%) follow-up. In fact, it has either the same or slightly higher sensitivity to predict biochemical recurrence as the combined HA-HYAL-1 staining inference (84% at 72-month and 81.5% at 112-month follow-up). The specificity of HYAL-1 staining inference (80.5%) at 72-month follow-up was slightly lower than that of HA-HYAL-1 (87.8%). However, HYAL-1 and HA-HYAL-1 staining inferences had same specificity (94.4%) to predict biochemical recurrence at ≥112 month follow-up. Therefore, HYAL-1 either has the same or slightly better PPV and NPV to predict biochemical recurrence. Thus, contrary to our earlier report that combined HA-HYAL-1 has slightly better prognostic capability than HYAL-1 staining alone at 64 months (12), our present study suggests that HYAL-1 alone is sufficiently accurate to predict biochemical recurrence ≥72 months.

In the multivariate analysis, among all of the pre- and post-operative parameters and histologic markers only pre-operative PSA and EPE had additional prognostic significance, if HYAL-1 (or HA-HYAL-1) was included in the analysis. In an earlier study we found that

EPE, margin status and HYAL-1 (or HA-HYAL-1) were independent predictors of prognosis. The difference between that study and this study is that in the earlier study we used 64 month follow-up as a cut-off limit and performed Wald's forward stepwise regression analysis, whereas, in this study we used the Cox Proportional hazard model and step-wise selection to calculate the hazard of biochemical recurrence over the entire period of follow-up (i.e., up to 131 month). Interestingly, when pre-operative PSA is not included in the model, margin status reaches independent prognostic significance together with EPE and HYAL-1 (or HA-HYAL-1), suggesting that pre-operative PSA provides all of the prognostic information related to margin status plus some additional prognostic information. Nonetheless, HYAL-1 appears to be an independent prognostic indicator for predicting biochemical recurrence.

As is the case for HA expression in tumor tissues, the prognostic significance of HYAL-1 expression may also vary based on the origin of cancer tissue. For example, in bladder cancer HYAL-1 expression correlates with tumor grade (21). It has also been shown that HAase levels are elevated in brain metastases of carcinomas when compared with primary glioblastomas (53). Furthermore, brain metastasis-derived cell lines have 1000-fold more HAase than glioma-derived cell lines (53). HYAL-1 levels are also elevated in the saliva of patients with squamous cell carcinoma of head and neck (24). However, HYAL-1 levels are not elevated in malignant ovarian tumors (54). Similarly, HYAL-1 expression suppresses tumor growth in a rat colon carcinoma model (55). However, HYAL-1 expression in PC-3 cells (a PCa cell line) increases their metastatic potential (56). Thus, HYAL-1 expression is associated with PCa progression.

The major dilemma for clinicians in the management of CaP is the identification of the site of disease recurrence, which ultimately guides therapy decisions. It is generally accepted that PSA recurrence of less than 1 to 2 years relates to a higher risk of developing metastatic disease (57,58). However, it is not understood whether existing pre- and post-operative parameters, as well as, histologic markers can distinguish between those patients who will recur in < 24 months and those who will not. In this study PSA sub-set analysis showed that except for margin status none of the markers (i.e., HA, HYAL-1, HA-HYAL-1, MVD and CD44v6) and pre- (i.e., age, clinical stage and pre-operative PSA) and post-operative (i.e., Gleason sum (overall or $\geq$7), EPE, seminal vesicle invasion) were able to distinguish between those patients who recurred in < 24 months and those who did not. It should be noted that in this cohort we had 25 patients who recurred in < 72 months, out of which 17 (68%) recurred within 24 months and 3 more recurred at 27 months (mean time to recur for the entire cohort was 21.3 months). Thus, the ability of various clinical and pathologic parameters, as well as, histologic markers to predict biochemical recurrence within 24 months may need to be studied in a larger group of biochemically recurred patients.

Nearly two thirds of PCa patients have preoperative PSA levels between 4 and 10 ng/ml, have a stage T1C disease and a biopsy Gleason between 5 and 7 (2,10,11). For such patients, one or a combination of accurate prognostic indicators could improve the physicians' ability to identify those PCa that are aggressive and will progress, so that individualized treatments could be offered. In this study, although pre-operative PSA was an independent predictor for prognosis, neither the overall Gleason sum nor Gleason sum categorized as, $\leq$and $\geq$7 was not an independent predictor for prognosis. Among the 4 potential prognostic indicators for PCa that we compared, HYAL-1 appears to an accurate and independent predictor for prognosis.

Acknowledgments: Department of Defense grant DAMD 170210005; NCI grant RO1 CA 072821-06A2; American Cancer Society Florida Division; Dr. Sinan Ekici was a fellow of The Scientific and Technical Council of Turkey (NATO Science Fellowship Program).

REFERENCES:

1. Khan, MA, Han, M, Partin, AW, Epstein, JI, Walsh, PC. Long-term cancer control of radical prostatectomy in men younger than 50 years of age: update 2003. Urology. 2003, 62: 86-91; discussion 91-2.
2. Draisma, G, Boer, R, Otto, SJ, van der Cruijsen, IW, Damhuis, RA, Schroder, FH, de Koning, HJ Lead times and overdetection due to prostate-specific antigen screening: estimates from the European Randomized Study of Screening for Prostate Cancer. J Natl Cancer Inst. 2003, 95: 868-878.
3. Palisaan RJ, Graefen M, Karakiewicz PI, Hammerer PG, Huland E, Haese A, Fernandez S, Erbersdobler A, Henke R.P, Huland H. Assessment of clinical and pathologic characteristics predisposing to disease recurrence following radical prostatectomy in men with pathologically organ-confined prostate cancer. Eur. Urol., 41: 155-161, 2002.
4. Pound CR, Partin AW, Epstein JI, Walsh PC. Prostate-specific antigen after anatomic radical retropubic prostatectomy. Patterns of recurrence and cancer control. Urol. Clin. North Am., *24:* 395-406, 1997.
5. Neulander, EZ, Soloway, MS. Failure after radical prostatectomy. Urology. 2003, 61: 30-36.
6. Symon, Z, Griffith, KA, McLaughlin, PW, Sullivan, M, Sandler, HM. Dose escalation for localized prostate cancer: substantial benefit observed with 3D conformal therapy. Int J Radiat Oncol Biol Phys. 2003, 57: 384-90.
7. Syed, S, Petrylak, DP, Thompson, IM. Management of high-risk localized prostate cancer: the integration of local and systemic therapy approaches. Urol Oncol. 2003, 21: 235-243.
8. Moul, JW. Variables in predicting survival based on treating "PSA-Only" relapse. Urol Oncol. 2003, 21: 292-304.
9. Swindle, PW, Kattan, MW, Scardino, PT. Markers and meaning of primary treatment failure. Urol Clin North Am. 2003, 30: 377-401.
10. Blute, ML, Bergstralh, EJ, Iocca, A, Scherer, B, Zincke, H. Use of Gleason score, prostate specific antigenm seminal vesicle and margin status to predict biochemical failure after radical prostatectomy. J. Urol., 165: 119-125, 2001.
11. Pettaway, CA. Prognostic markers in clinically localized prostate cancer. Tech. Urol., 4: 35-42, 1998.
12. Posey, JT, Soloway, MS, Ekici, S, Sofer, M, Civantos, F, Duncan, RC., Lokeshwar, VB. Evaluation of the prognostic potential of hyaluronic acid and hyaluronidase (HYAL1) for prostate cancer. Cancer Res 63:2638-2644, 2003.
13. Tammi MI. Day AJ, Turley EA. Hyaluronan and homeostasis: a balancing act. J. Biol. Chem., *277:* 4581-4584, 2002.
14. Turley EA, Noble PW, Bourguignon LY Signaling properties of hyaluronan receptors. J. Biol. Chem., *277:* 4589-4592, 2002.
15. Setala LP, Tammi MI, Tammi RH, Eskelin MJ, Lipponen PR, Argen UM, Parkkinen J, Alhava EM, Kosma VM. Hyaluronan expression in gastric cancer cells is associated with local and nodal spread and reduced survival rate. Br. J. Cancer, *79:* 1133-1138, 1999.
16. Auvinen P, Tammi R, Parkkinen J, Tammi M, Agren U, Johansson R, Hirvikoski P, Eskelinen M, Kosma VM. Hyaluronan in peritumoral stroma and malignant cells associates with breast cancer spreading and predicts survival. Am. J. Pathol., *156:* 529-536, 2000.
17. Knudson W. Tumor associated hyaluronan: providing an extracellular matrix that facilitates invasion. Am. J. Pathol., *148:* 1721-1726, 1996.
18. Ropponen K, Tammi M, Parkkinen J, Eskelinen M, Tammi R, Lipponen P, Argen V, Alhava E, Kosma VM. Tumor-associated hyaluronan as an unfavorable prognostic factor in colorectal cancer. Cancer Res., *58:* 342-347, 1998.

19. Lipponen P, Aaltomaa S, Tammi R, Tammi M. Agren U, Kosma VM. High stromal hyaluronan level is associated with poor differentiation and metastasis in prostate cancer. Eur. J. Cancer, *37:* 849-856, 2001.
20. Lokeshwar VB, Obek C, Pham HT, Wei DC, Young MJ, Duncan RC, Soloway MS, Block NL. Urinary hyaluronic acid and hyaluronidase: markers for bladder cancer detection and evaluation of grade. J. Urol., *163:* 348-356, 2000.
21. Hautmann SH, Lokeshwar VB, Schroeder GL, Civantos F, Duncan RC, Friedrich MG, Soloway MS. Elevated tissue expression of hyaluronic acid and hyaluronidase validate HA-HAase urine test for bladder cancer. J. Urol., *165:* 2068-2074, 2001.
22. Lokeshwar VB, Rubinowicz D, Schroeder GL, Forgacs E, Minna JD, Block NL, Nadji. M, Lokeshwar BL. Stromal and epithelial expression of tumor markers hyaluronic acid and hyaluronidase in prostate cancer. J. Biol. Chem., *276:* 11922-11932, 2001.
23. Csoka, AB, Frost, GI, Stern, R. The six hyaluronidase-like genes in the human and mouse genomes. Matrix Biol. 20: 499-508, 2001.
24. Franzmann, EJ, Schroeder, GL, Goodwin, WJ, Weed, DT, Fisher, P, Lokeshwar, VB. Expression of tumor markers hyaluronic acid and hyaluronidase (HYAL1) in head and neck tumors. Int J Cancer. 2003, 106: 438-445.
25. West DC, Hampson IN, Arnold F, Kumar S. Angiogenesis induced by degradation products of hyaluronic acid. Science (Wash. DC), *228:* 1324-1326, 1985.
26. Lokeshwar VB, Selzer MG. Differences in hyaluronic acid-mediated functions and signaling in arterial, microvessel, and vein-derived human endothelial cells. J. Biol. Chem., *275:* 27641-27649, 2000.
27. Pham, HT, Block, NL, Lokeshwar, VB. Tumor derived hyaluronidase: a diagnostic urine marker for high-grade bladder cancer. Cancer Res., 57: 778-783, 1997.
28. Lesley, J, Hascall, VC, Tammi, M, Hyman, R. Hyaluronan binding by cell surface CD44. J Biol Chem. 2000 275: 26967-26975.
29. Cichy, J, Pure, E. The liberation of CD44. J Cell Biol. 2003, 161: 839-843.
30. Naor, D, Nedvetzki, S, Golan, I, Melnik, L, Faitelson, Y. CD44 in cancer. Crit Rev Clin Lab Sci. 2002, 39: 527-579.
31. Lokeshwar, VB, Fregien, N, Bourguignon, LY. Ankyrin-binding domain of CD44(GP85) is required for the expression of hyaluronic acid-mediated adhesion function. J Cell Biol. 1994 126: 1099-1109.
32. Lokeshwar, VB, Iida, N, Bourguignon, LY. The cell adhesion molecule, GP116, is a new CD44 variant (ex14/v10) involved in hyaluronic acid binding and endothelial cell proliferation. J Biol Chem. 1996, 271: 23853-23864.
33. Lokeshwar, BL, Lokeshwar, VB, Block, NL. Expression of CD44 in prostate cancer cells: association with cell proliferation and invasive potential. Anticancer Res. 1995, 15: 1191-118.
34. Welsh, CF, Zhu, D, Bourguignon, LY. Interaction of CD44 variant isoforms with hyaluronic acid and the cytoskeleton in human prostate cancer cells. J Cell Physiol. 1995, 64: 605-612.
35. Iczkowski, KA, Bai, S, Pantazis, CG. Prostate cancer overexpresses CD44 variants 7-9 at the messenger RNA and protein level. Anticancer Res. 2003 234: 3129-3140.
36. Gao, AC., Lou, W., Sleeman, JP, Isaacs, JT. Metastasis suppression by the standard CD44 isoform does not require the binding of prostate cancer cells to hyaluronate. Cancer Res. 1998, 58: 2350-2352.
37. Ekici, S, Ayhan, A, Kendi, S, Ozen, H. Determination of prognosis in patients with prostate cancer treated with radical prostatectomy: prognostic value of CD44v6 score. J Urol, 167: 2037-2041, 2002.
38. Aaltomaa, S, Lipponen, P, Ala-Opas, M. Kosma, VM. Expression and prognostic value of CD44 standard and variant v3 and v6 isoforms in prostate cancer. Eur Urol., 2001 39:138-144.
39. Sato, Y. Molecular diagnosis of tumor angiogenesis and anti-angiogenic cancer therapy. Int J Clin Oncol. 2003, 8: 200-206.

40. Sullivan, DC, Bicknell, R. New molecular pathways in angiogenesis. Br J Cancer. 2003, 89: 228-231.
41. Folkman, J, Browder, T, Palmblad, J. Angiogenesis research: guidelines for translation to clinical application. Thromb Haemost. 2001, 86: 23-33.
42. Sauer, G, Deissler, H. Angiogenesis: prognostic and therapeutic implications in gynecologic and breast malignancies. Curr Opin Obstet Gynecol. 2003, 15: 45-49.
43. Goddard, JC, Sutton, CD, Furness, PN, O'Byrne, KJ, Kockelbergh, R.C. Microvessel density at presentation predicts subsequent muscle invasion in superficial bladder cancer. Clin Cancer Res. 2003, 9: 2583-2586.
44. Poon, RT, Fan, ST, Wong, J. Clinical significance of angiogenesis in gastrointestinal cancers: a target for novel prognostic and therapeutic approaches. Ann Surg. 2003, 238: 9-28.
45. Zhang, X, Yamashita, M, Uetsuki, H, Kakehi, Y. Angiogenesis in renal cell carcinoma: Evaluation of microvessel density, vascular endothelial growth factor and matrix metalloproteinases. Int J Urol. 2002, 9: 509-514.
46. De La Taille, A, Katz, AE, Bagiella, E, Buttyan, R, Sharir, S, Olsson, CA, Burchardt, T, Ennis, RD, Rubin, MA. Microvessel density as a predictor of PSA recurrence after radical prostatectomy : A comparison of CD34 and CD31. Am J Clin Pathol 2000, 113: 555-562.
47. Bono, AV, Celato, N., Cova, V., Salvadore, M., Chinetti, S., Novario, R. Microvessel density in prostate carcinoma. Prostate Cancer Prostatic Dis. 2002, 5: 123-127.
48. Weidner, N., Carroll, P.R., Flax, J., Blumenfeld, W., Folkman, J. Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. Am J Pathol 1993, 143: 401-409.
49. Silberman, M.A., Partin, A.W., Veltri, R.W., Epstein, J.I. Tumor angiogenesis correlates with progression after radical prostatectomy but not with pathologic stage in Gleason sum 5 to 7 adenocarcinoma of the prostate. Cancer 1997, 79: 772-779.
50. Gettman, M.T., Bergstralh, E.J., Blute, M., Zincke, H., Bostwick, D.G. Prediction of patient outcome in pathologic stage T2 adenocarcinoma of the prostate: Lack of significance for microvessel density analysis. Urology 1998, 51:79-85.
51. Rubin, M.A., Buyyounouski, M., Bagiella, E., Sharir, S., Neugut, A., Benson, M., De La Taille, A., Katz, A.E., Olsson, C.A., Ennis, R.D. Microvessel density in prostate cancer : Lack of correlation with tumor grade, pathologic stage, and clinical outcome. Urology 1999, 53: 542-547.
52. Norrby, K. Ridell, B. Tumour-type-specific capillary endothelial cell stainability in malignant B-cell lymphomas using antibodies against CD31, CD34 and Factor VIII. Br J Cancer, 2002, 87: 694-701.
53. Delpech, B., Laquerriere, A., Maingonnat, C., Bertrand, P., Freger, P. Hyaluronidase is more elevated in human brain metastases than in primary brain tumours. Anticancer Res. 2002, 22: 2423-2427.
54. Hiltunen, E.L., Anttila, M., Kultti, A., Ropponen, K., Penttinen, J., Yliskoski, M., Kuronen, A.T., Juhola, M., Tammi, R., Tammi, M., Kosma, V.M. Elevated hyaluronan concentration without hyaluronidase activation in malignant epithelial ovarian tumors. Cancer Res., 2002, 62: 6410-6413.
55. Jacobson, A., Rahmanian, M., Rubin, K., Heldin, P. Expression of hyaluronan synthase 2 or hyaluronidase 1 differentially affect the growth rate of transplantable colon carcinoma cell tumors. Int J Cancer. 2002, 102: 212-219.
56. Patel, S., Turner, P.R., Stubberfield, C., Barry, E., Rohlff, C.R., Stamps, A., McKenzie, E., Young, K., Tyson, K., Terrett, J., Box, G., Eccles, S., Page, M.J. Hyaluronidase gene profiling and role of hyal-1 overexpression in an orthotopic model of prostate cancer. Int J Cancer. 2002, 97: 416-424.
57. Swindle, P.W., Kattan, M.W., Scardino, P.T. Markers and meaning of primary treatment failure. Urol Clin North Am., 2003, 30 : 377-401.
58. Canto, E.I., Shariat, S.F., Slawin, K.M. Biochemical staging of prostate cancer. Urol Clin North Am. 2003, 30: 263-277.

Table 1: Pre- and postoperative parameters of the study patients

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 Mean: 65.1	Median: 9.0 Mean: 14.04	T1c: 10 T2a: 5 T2b: 10	6 = 2 7 = 14 8 = 6 9 = 3	(+) = 21 (-) = 4	(+)=18 (-)=7	(+) = 14 (-) = 11
No biochemical or clinical recurrence (n = 41)	Median: 65 Mean: 62.98	Median: 6 Mean: 8.1	T1c: 22 T2a: 5 T2b: 14	5 = 7 6 = 9 7 = 20 8 = 5	(+) = 4 (-) = 37	(+)=9 (-)=32	(+) = 3 (-) = 38

Table 2: Sensitivity, specificity, accuracy, PPV and NPV of HA, HYAL-1, combined HA-HYAL-1, CD34 and CD44v6 staining inferences. Note that 72 months, 84 months, 100 months and 112 months were used a cut point for determining biochemical recurrence.

Table 2 A: HA, HYAL-1 and HA-HYAL-1

	HA (%)				HYAL-1 (%)				HA-HYAL-1 (%)			
	months		months	months	months		months	months	months	months	months	
Sensitivity	96 (24/25)	96 (24/25)	92.3 (24/26)	92.6 (25/27)	84 (21/25)	84 (21/25)	84.6 (22/26)	85.2 (23/27)	84 (21/25)	84 (21/25)	80.8 (21/26)	81.5 (22/27)
Specificity	61 (25/41)	61.1 (22/36)	65.4 (17/26)	80.6 (16/18)	80.5 (33/41)	80.6 (29/36)	84.6 (22/26)	94.4 (17/18)	87.8 (36/41)	88.9 (32/36)	88.5 (23/26)	94.4 (17/18)
Accuracy	74.2 (49/66)	75.4 (46/61)	78.8 (41/52)	91.1 (41/45)	81.8 (54/66)	82 (50/61)	84.6 (44/52)	88.9 (40/45)	86.4 (57/66)	86.9 (53/61)	84.6 (44/52)	86.7 (39/45)
PPV	60 (24/40)	63.2 (24/38)	77.4 (24/31)	92.6 (25/27)	70 (21/30)	75 (21/28)	84.6 (22/26)	95.8 (23/24)	80.8 (21/26)	84 (21/25)	87.5 (21/24)	95.7 (22/23)
NPV	96.1 (25/26)	95.7 (22/23)	89.5 (17/19)	80.6 (16/18)	89.3 (34/37)	87.9 (29/33)	84.6 (22/26)	81 (17/21)	90 (36/40)	88.9 (32/36)	85.2 (23/27)	77.3 (17/22)

Table 2 B: CD34 and CD44v6

	MVD (%)				CD44v6 (%)			
Parameters	72 months	84 months	100 months	112 months	72 months	84 months	100 months	112 months
Sensitivity	76 (19/25)	76 (19/25)	76.9 (20/26)	77.8 (21/27)	68 (17/25)	68 (17/25)	65.4 (17/26)	62.9 (17/27)
Specificity	61 (25/41)	61.1 (22/36)	65.4 (17/26)	77.8 (14/18)	56.1 (23/41)	52.8 (19/36)	50 (13/26)	61.1 (11/18)
Accuracy	66.7 (44/66)	67.2 (41/61)	71.1 (37/52)	77.8 (35/45)	57.6 (38/66)	59 (36/61)	57.7 (30/52)	62.2(28/45)
PPV	54.3 (19/35)	57.6 (19/33)	69 (20/29)	84 (21/25)	48.6 (17/35)	50 (17/34)	56.7 (17/30)	70.8 (17/24)
NPV	80.6 (25/31)	78.6 (22/28)	73.4 (17/23)	70 (14/20)	74.2 (23/31)	70.4 (19/27)	59 (13/22)	52.4 (11/21)

Table 3: Univariate analysis of pre- and post-operative prognostic parameters and IHC staining inferences. *: Statistically significant. CI: Confidence Interval. Cox proportional Hazard model and single parameter analysis was used to determine the prognostic significance of pre-operative (age, pre-operative PSA, and clinical stage) and post-operative (Gleason sum (overall) or Gleason sum categorized ($\geq$ and $\leq$ 7), margin +/-, EPE +/-, seminal vesicle invasion +/-) parameters and HA, HYAL-1, HA-HYAL-1, MVD and CD44v6 staining inferences.

Parameter	χ^2	p value	Hazard ratio	95% CI*
Age	0.4332	0.5104	1.019	0.963 – 1.078
PSA	11.648	0.0006*	1.048	1.02 – 1.077
Gleason sum (overall)	14.19	0.0002*	2.5	1.552 – 4.024
Gleason $\geq$ 7	5.744	0.0165*	5.827	1.379 – 24.633
Clinical stage	1.226	0.2683	1.262	0.836 – 1.905
EPE	25.411	< 0.0001	12.781	4.746 – 34.42
Surgical margin positivity	13.355	0.0003*	4.5	2.008 – 10.079
Seminal vesicle invasion	22.268	< 0.0001	6.56	3.002 – 14.317
HA	11.319	0.0008*	12.091	2.831 – 51.648
HYAL-1	22.054	<0.0001*	13.192	4.5 – 38.716
HA-HYAL-1	25.364	<0.0001*	10.749	4.266 – 27.087
MVD	10.0314	0.0015*	4.36	1.753 – 10.845
CD44v6	2.277	0.131	1.826	0.835 – 3.994

Table 4: Multivariate analysis of pre- and post-operative prognostic parameters and IHC staining inferences. Cox Proportional Hazard model and stepwise selection was used to determine which of the pre-operative (i.e., age, PSA and clinical stage) and post-operative (i.e., Gleason sum, Gleason sum categorized (≥and ≤7), EPE, margin +/-, and seminal vesicle invasion) parameters and HA, HYAL-1, HA-HYAL-1, MVD and CD44v6 staining inferences have independent prognostic significance. The significant parameters ($P > 0.05$) selected by the model are shown. **A:** In the analysis, HA and HYAL-1staining inferences were included separately in the model. **B:** Combined HA-HYAL-1 staining inference was included in the analysis. NA: Not applicable. *: Statistically significant.

Table 4 A				
Parameters	χ^2	p value	Hazard ratio	95% CI*
PSA	16.857	< 0.0001*	1.086	1.044 – 1.130
EPE	9.939	0.0016*	6.222	1.997 – 19.384
HYAL-1	11.094	0.0009*	8.196	2.377 – 26.259
Table 4 B				
PSA	14.127	0.0002*	1.077	1.036 – 1.12
EPE	10.998	0.0009*	6.906	2.204 – 21.640
HA-HYAL-1	9.428	0.0021*	5.191	1.814 – 14.854

Table 5: PSA sub-group analysis. The ability of PSA and age to predict PSA recurrence within 24 months was determined using the t test (since these were continuous variables). Mantel-Haenszel chi-square analysis was used to evaluate the ability of clinical stage, post-operative parameters and IHC staining markers to predict PSA recurrence within 24 months.

Parameter	**t value**	**p value**
Age	0.66	0.513
PSA	1.06	0.298
Parameter	χ^2	**p value**
Gleason sum (overall)	0.133	0.453
Gleason ≥ 7	0.15	0.699
Clinical stage	1.903	0.168
EPE	0.736	0.391
Surgical margin positivity	4.894	0.0269*
Seminal vesicle invasion	0.122	0.7265
HA	0.15	0.699
HYAL-1	0.280	0.596
HA-HYAL-1	0.0437	0.834
MVD	1.322	0.25
CD44v6	1.102	0.294

Figure Legend:

Figure 1: Localization of HA, HYAL-1, CD44v6 and MVD in PCa tissues: Histologic markers were localized in PCa tissues from a non-progressed patient (panels A, C, E, G) and a progressed patient (panels B, D, F, H). Panels A and B: HA was localized in PCa tissues using a biotinylated HA-binding protein. Panels C and D: HYAL-1 was localized in PCa tissues using an anti-HYAL-1 antibody. Panels E and F: CD44v6 was localized in PCa tissues using an anti-CD44v6 monoclonal antibody. Panels G and H: MVD was visualized using an anti-CD34 monoclonal antibody. The each panel represents 400X magnification.

Figure 2: Kaplan-Meier analysis. Kaplan-Meier analysis was performed after stratifying the data as HYAL-1 high (H)/Low (L), EPE +/- and PSA < or < 7 ng/ml. **A:** Kaplan-Meier analysis of data stratified as HYAL1 H/L and EPE +/-. **B:** Kaplan-Meier analysis of data stratified as HYAL-1 H/L and PSA > or < 7 ng/ml.

FIGURE 1: Ekici et al

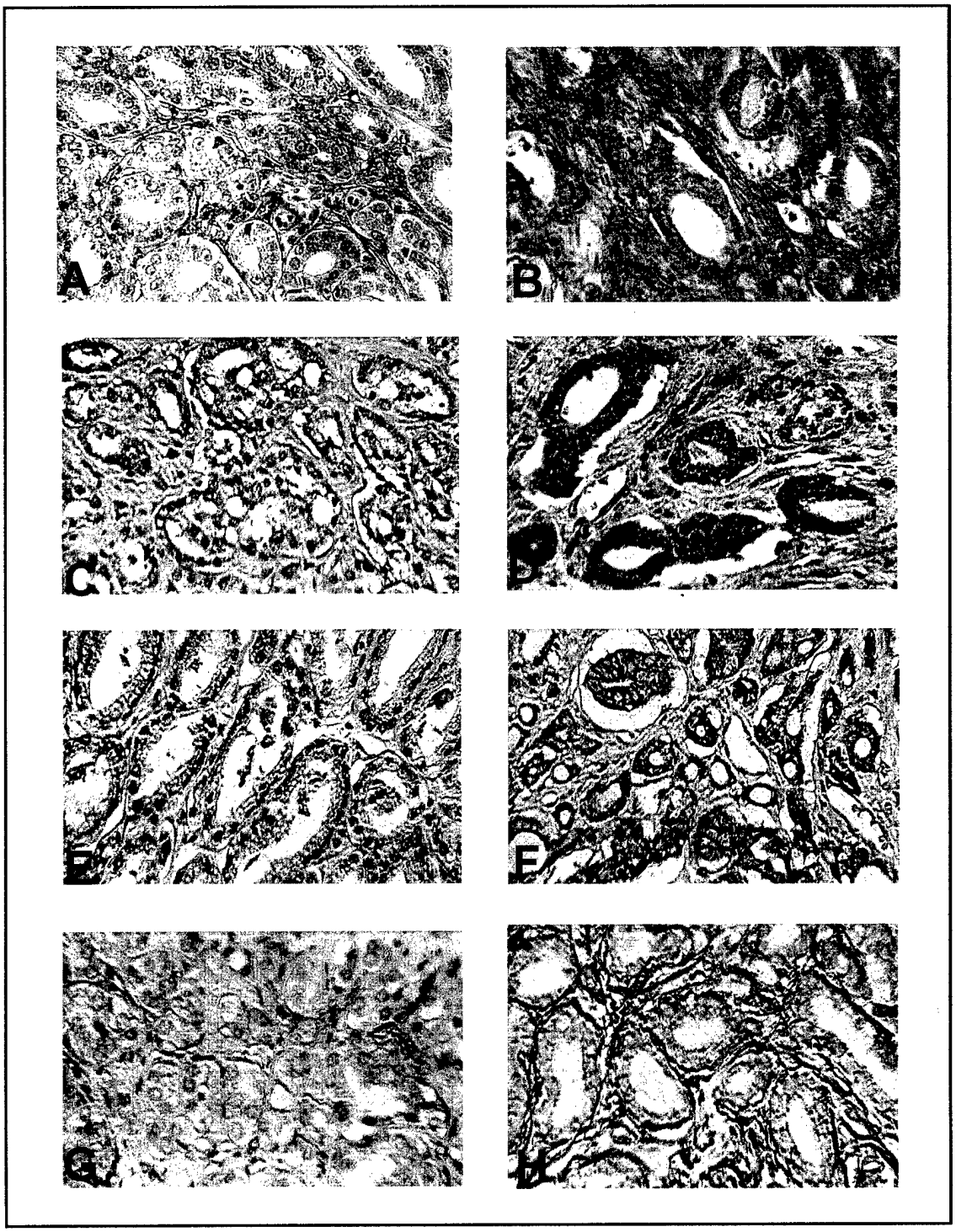


Fig 2 Ekici et al