



Bladder Cancer

HYAL-1 Hyaluronidase: A Potential Prognostic Indicator for Progression to Muscle Invasion and Recurrence in Bladder Cancer

Mario W. Kramer^{a,b}, Roozbeh Golshani^d, Axel S. Merseburger^{b,c}, Judith Knapp^b, Alfredo Garcia^a, Joerg Hennenlotter^b, Robert C. Duncan^e, Mark S. Soloway^a, Merce Jorda^f, Marcus A. Kuczyk^{b,c}, Arnulf Stenzl^b, Vinata B. Lokeshwar^{g,*}

^a Department of Urology, University of Miami Miller School of Medicine, Miami, FL, USA

^b Department of Urology, Eberhard-Karls-University Tübingen, Tübingen, Germany

^c Current location: Department of Urology, Medizinische Hochschule Hannover (MHH), Hannover, Germany

^d Department of Cell Biology and Anatomy, University of Miami Miller School of Medicine, Miami, FL, USA

^e Department of Epidemiology and Public Health, University of Miami Miller School of Medicine, Miami, FL, USA

^f Department of Pathology, University of Miami Miller School of Medicine, Miami, FL, USA

^g Department of Urology, Cell Biology and Anatomy, Sylvester Comprehensive Cancer Center, University of Miami Miller School of Medicine, Miami, FL, USA

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Abstract

Background: For bladder cancer (BCa) patients undergoing bladder-sparing treatments, molecular markers may aid in accurately predicting progression to muscle invasion and recurrence. Hyaluronic acid (HA) is a glycosaminoglycan that promotes tumor metastasis. Hyaluronoglucosaminidase 1 (HYAL-1)-type hyaluronidase (HAase) promotes tumor growth, invasion, and angiogenesis. Urinary HA and HAase levels are diagnostic markers for BCa.

Objective: We evaluated whether HA and HYAL-1 can predict progression to muscle invasion and recurrence among patients with non-muscle-invasive BCa.

Design, setting, and participants: Based on tissue availability, tissue microarrays were prepared from a cohort of 178 BCa specimens (144 non-muscle invasive, 34 muscle invasive). Follow-up information was available on 111 patients with non-muscle-invasive BCa (mean follow-up: 69.5 mo); 58 patients recurred and 25 progressed to muscle invasion (mean time to progress: 22.3 mo).

Measurements: HA and HYAL-1 expression was evaluated by immunohistochemistry and graded for intensity and area of staining. Association of HA and HYAL-1 staining with BCa recurrence and muscle invasion was evaluated by univariate and multivariate models.

Results and limitations: HA and HYAL-1 expression correlated with tumor grade, stage, and multifocality ($p < 0.05$). In non-muscle-invasive BCa specimens, HYAL-1 staining was higher (234.3 ± 52.2 ; 200.6 ± 61.4) if patients experienced progression to muscle invasion or recurrence when compared with no progression or recurrence (164.1 ± 48.2 ; 172.1 ± 57 ; $p < 0.001$). HA staining correlated with muscle invasion ($p < 0.001$). In univariate analysis, age ($p = 0.014$), multifocality ($p = 0.023$), and HYAL-1 staining ($p < 0.001$) correlated with muscle invasion, whereas only HYAL-1 correlated with recurrence ($p = 0.013$). In multivariate analysis, HYAL-1 significantly associated with muscle invasion ($p < 0.001$; 76.8% accuracy) and recurrence ($p = 0.01$; 67.8% accuracy).

Conclusions: HYAL-1 is a potential prognostic marker for predicting progression to muscle invasion and recurrence.

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* Corresponding author. Department of Urology (M-800), University of Miami Miller School of Medicine, P.O. Box 016960, Miami, Florida 33101, USA. Tel: +305 243 6321; Fax: +1 305 243 6893. E-mail address: vlokeshw@med.miami.edu (V.B. Lokeshwar).

1. Introduction

Patients with non-muscle-invasive bladder cancer (BCa; ie, stage Ta, T1, or carcinoma in situ [CIS]) are often treated with bladder-sparing treatments. Current clinical and pathologic parameters, such as tumor grade, stage, and multifocality, are well investigated in terms of providing prognostic information regarding progression to muscle invasion and recurrence. Additional markers, however, may help in making better prognostic predictions. Prognostic information for patients undergoing bladder-sparing treatments is important, since these patients are at risk for developing new tumors in the bladder (ie, recurrence) and for disease progression to stage T2 or higher. Furthermore, if a patient fails a bladder-sparing treatment, the tumor could become muscle invasive during the course of the treatment. Several markers associated with BCa growth and progression have been tested as prognostic markers [1–3].

Hyaluronic acid (HA) is a nonsulfated glycosaminoglycan that promotes tumor metastasis. HA levels are elevated in a variety of tumors [4,5]. We have shown that the measurement of urinary HA levels by HA test is approximately 85% accurate in detecting BCa [6]. We recently demonstrated that inhibition of HA synthesis in BCa cells inhibits tumor growth, invasion, and angiogenesis [7,8]. Hyaluronoglucosaminidase 1 (HYAL-1) is the major hyaluronidase expressed in BCa cells and is an accurate marker for high-grade BCa [6,9,10]. We have shown that inhibition of HYAL-1 expression in tumor cells decreases tumor growth, invasion, and angiogenesis [11].

Because HA and HYAL-1 promote BCa growth and progression, we investigated whether these molecules have prognostic significance for predicting disease progression and bladder tumor recurrence.

2. Materials and methods

2.1. Patient characteristics

Archival BCa specimens ($n = 178$, from 178 patients), collected between 1993 and 2001, were obtained from the University of Tübingen in Germany and the University of Miami in the United States. Of those, 144 specimens were from patients with non-muscle-invasive BCa (stage Ta, T1) and 34 were from patients with muscle-invasive BCa (stages T2, T3, or T4). CIS was concomitantly present in eight patients with T1 disease. The presence of CIS was established by random biopsies based on the surgeons' impression. The study was approved by the institutional review boards at both institutions. Patient and tumor characteristics are detailed in Table 1. In this study, specimen selection was based solely on the availability of well-preserved tumor tissue blocks ($n = 227$) and the availability of enough tissue in those blocks for microarray preparation ($n = 190$). Of the 190 specimens, 12 could not be evaluated due to the loss of tissue during microarray preparation or staining.

Non-muscle-invasive patients ($n = 144$) were treated with transurethral resection of bladder tumor (TURBT); 78 of these patients underwent TURBT alone and 66 received intravesical therapy following TURBT. Follow-up information was available for 111 patients; 58 experienced tumor recurrence and 25 showed progression to muscle invasion. Recurrence was defined as detection of bladder tumor by cystoscopy after treatment (TURBT with or without intravesical

therapy). Progression to muscle invasion was defined as the detection of a tumor stage T2 or higher in the bladder after treatment (ie, recurrence of a stage T2 or higher tumor). The mean and median time of total follow-up, time to recur, and time to progression to muscle invasion were computed from Kaplan-Meier plots. Of the 34 patients with muscle-invasive disease, follow-up information was available for 31 patients (10 developed metastasis and 21 were free of metastasis; Table 1).

2.2. Tissue microarray preparation

Tissue microarrays (TMAs) were prepared as previously described [12]. A hematoxylin and eosin (H&E) slide of each archival specimen was evaluated, and a block representing the overall tumor grade (donor block) was chosen for TMA preparation. Three 0.6-mm core biopsies were punched from the donor block and transferred to a paraffin block (TMA block). Each TMA block had a total of 75 core biopsies (ie, 25 specimens from 25 BCa patients). Sections from the TMA blocks were cut 4- μ m thick and placed on positively charged slides for immunostaining. Each H&E slide of each TMA was evaluated by the study pathologist (MJ) to ensure that the 0.6-mm core was representative of the tumor grade of the specimen.

2.3. Immunohistochemistry

TMA slides were sequentially deparaffinized, rehydrated, and subjected to antigen retrieval. The slides were incubated with a biotinylated HA binding protein (1 μ g/ml; for HA staining) or an anti-HYAL-1 immunoglobulin G (1 μ g/ml) at 4 °C for 15 h [8,13–17]. We have previously described the nature and specifics of these primary detection reagents [8,13–17]. The positive and negative controls for HYAL-1 immunohistochemistry were tumor xenograft specimens of HT1376 human BCa cells transfected with HYAL-1 sense complementary DNA (cDNA) and HYAL-1 antisense cDNA [11]. For HA immunohistochemistry we used the tumor xenograft specimens of HT1376 cells transfected with HAS-1 sense cDNA (positive control) or HAS-1 cDNA (negative control) [7]. After incubation with primary reagents, the slides were sequentially processed using the Dako LSAB kit (Dako, Glostrup, Denmark) and 3,3'-diaminobenzidine substrate solution, and then were counterstained with hematoxylin.

2.4. Slide grading

Two researchers (MWK and RG) graded all slides independently and in a blinded fashion when both researchers were present at one institution (University of Miami). Each core was graded for staining intensity (0–3+) and then multiplied by the area of staining; thus, each specimen could receive a staining score between 0 and 300. The intensity scores of the three cores from the same specimen were averaged to obtain the mean intensity score for a specimen for each reader. The mean intensity scores of the two readers were then averaged to get the final score [12,18]. If the average staining scores of the two readers differed by $\geq 20\%$ (eg, score 220 for reader 1 versus 180 for reader 2) for a specimen, the cores of that specimen were reevaluated by the same two readers and by a third reader (ASM, when he was on a research fellowship at the University of Miami). The slides were also evaluated using the IP image analysis software, and the results were comparable to the readers' scores. It is noteworthy that, overall, there was agreement of approximately 90% between the two readers with respect to scoring of all 178 specimens and between the average scores of the readers and the results of the IP image analysis software. This was further confirmed by Pearson correlation analysis. There was significant correlation between the staining scores of the two readers (Spearman ρ : 0.876; 95% confidence interval [CI]: 0.789–0.946; $p \leq 0.001$) and between the average scores of

Table 1 – Patient characteristics

Parameter			95% CI
Age, yr	Mean: 67.7	Median: 69	65.8–69.6
Gender			
Male	n = 132 (74.2%)	–	–
Female	n = 46 (25.8%)	–	–
Grade [*]			
G1	n = 51 (28.7%)	–	–
G2	n = 89 (50%)	–	–
G3	n = 38 (21.3%)	–	–
Stage			
Ta	n = 82 (46.1%)	–	–
T1	n = 62 (34.8%)	–	–
T2	n = 18 (10.1%)	–	–
T3	n = 13 (7.3%)	–	–
T4	n = 3 (1.7%)	–	–
Multifocality	n = 66 (36.9%)	–	–
Concomitant CIS	n = 8 (4.5%)	–	–
Node positive ^{**}	n = 21 (61.7%)	–	–
Non-muscle-invasive patients with follow-up (n = 111)			
Total follow-up, mo	Mean: 69.5	Median: 60	54–66
Recurrence			
Negative	n = 53 (47.7%)	–	–
Positive	n = 58 (52.1%)	–	–
Time to recurrence, mo	Mean: 14.9	Median: 11	7–12
Progression			
Negative	n = 86 (76.0%)	–	–
Positive	n = 25 (24%)	–	–
Time to progression, mo	Mean: 22.3	Median: 12	7–23
Muscle-invasive patients with follow-up (n = 31)			
Negative	n = 21 (67.7%)	–	–
Positive	n = 10 (32.3%)	–	–
Total follow-up, mo	Mean: 61.4	Median: 56.5	52–64
Time to metastasis, mo	Mean: 11.4	Median: 9.0	2–12

CI = confidence interval; CIS = carcinoma in situ.
^{*} In our study, the grade classification was based on the pathologic World Health Organization (WHO) 1973 classification. Since this study included tumors collected between 1993 and 2001, the pathologist had classified the tumors as per the WHO 1973 classification.
^{**} None of the patients with non-muscle-invasive bladder cancer were node positive; therefore, the percentage is calculated based on the number of patients with muscle-invasive disease (n = 34).

the two readers and the IP image analysis scores (Spearman ρ : 0.874; 95% CI: 0.798–0.953; $p < 0.001$). Because there is significant correlation between the manual evaluation of staining and the image analysis software, it may be possible to use image analysis software to evaluate HA or HYAL-1 staining in future multicenter studies.

2.5. Statistical analyses

The differences in the mean HA and HYAL-1 staining scores among various tumor grades or stages were calculated by Kruskal-Wallis test, assuming a non-normal distribution, followed by Dunn multiple comparison test. The differences in the mean HA and HYAL-1 staining scores with respect to sex, multifocality, and age (stratified at a median age of 69 yr) were calculated by Mann-Whitney test.

Because this is a retrospective cohort study, we initially tested whether the Cox proportional hazards model would be appropriate for determining the association of HA and/or HYAL-1 staining with progression to muscle invasion and recurrence in a multivariate model. We categorized HA and HYAL-1 staining inferences in tertiles and at the median. Plots of log hazard (log H) and log time showed approximately linear plots with constant separation between the groups for the tertile categorization and the median categorization, suggesting that the Cox model is appropriate. We then performed Cox proportional hazards

analysis by including all pre- and postoperative parameters listed in Table 1 and HA and HYAL-1 staining scores to determine the parameters that jointly predict tumor recurrence and/or progression to muscle invasion. Kaplan-Meier plots were generated for those parameters that reached statistical significance in the multivariate models.

Receiver operating curves were generated to determine the association between staining scores and tumor recurrence or progression to muscle invasion. Cut-off values selected by a statistical program (JMP 6 software; SAS Institute, Cary, NC, USA) were used for defining high or low expression of HA (cut-off: 175) and HYAL-1 (cut-off: 210). The program selected the cut-off limit that yielded the highest sensitivity (1-Specificity) value. A staining score greater than or equal to the cut-off value was considered to be a true positive if the patient had recurrence (or progression to muscle invasion); a score lower than the cut-off value was considered to be a true negative if the patient had no recurrence (or progression to muscle invasion). The sensitivity, specificity, and accuracy for HA and HYAL-1 staining inferences were calculated as previously described [8,13–15]. Cross-validation was performed to obtain the mean plus or minus the standard deviation (SD) and 95% CI for the sensitivity and specificity for HA and HYAL-1 staining scores, as previously described [18]. Statistical analyses were carried out using the JMP software program (version 6.0; SAS Institute).

3. Results

3.1. HA and HYAL-1 expression increases with tumor grade and stage

Analysis of HA and HYAL-1 staining in TMA specimens showed that HA and HYAL-1 staining intensity scores increased with tumor grade and stage ($p < 0.05$ for HA staining, $p < 0.01$ or $p < 0.001$ for HYAL-1 staining; Table 2). While the presence of multifocality correlated with HA and HYAL-1 staining scores, concomitant presence of CIS correlated only with HYAL-1 staining.

3.2. Correlation of HA and HYAL-1 staining scores with progression to muscle invasion and tumor recurrence

We next evaluated whether HA and or HYAL-1 staining in non-muscle-invasive specimens predicts muscle invasion and/or tumor recurrence. HA and HYAL-1 staining is low in TaG2 specimens from patients with non-muscle-invasive disease who did not recur (Fig. 1A, panels a and c). HA and HYAL-1 staining, however, is high in TaG2 specimens from patients who later recurred with muscle-invasive BCa (Fig. 1A, panels b and d). As we have reported previously, in these specimens, HA is localized in both the tumor-associated stroma and the tumor cells, whereas HYAL-1 is exclusively expressed in tumor cells. Fig. 1B shows that

among patients who progressed to muscle invasion and those who did not, the differences in the mean (plus or minus SD) staining intensity scores for both HA and HYAL-1 are statistically significant. Only the differences in the mean (plus or minus SD) HYAL-1 staining scores among those who recurred and those who did not are statistically significant.

To determine whether any of the pre- and postoperative parameters and/or HA and HYAL-1 staining inferences predict progression to muscle invasion and/or recurrence, we performed logistic regression analysis (univariate analysis). As shown in Table 3, age, multifocality, and HYAL-1 staining inferences correlate with muscle invasion, whereas only HYAL-1 staining score significantly correlates with tumor recurrence.

3.3. HYAL-1 staining inferences independently correlate with tumor recurrence and progression to muscle invasion

To determine which of the pre- and postoperative parameters and/or HA and HYAL-1 staining inferences are independent prognostic predictors of tumor recurrence and/or muscle invasion, we used the Cox proportional hazards model. When only pre- and postoperative parameters were included in the model (ie, no HA and HYAL-1 in the model), no clinical or pathologic parameter was significant in predicting recurrence and progression to muscle invasion. When both HA and HYAL-1 were included

Table 2 – Distribution of hyaluronic acid (HA) and hyaluronoglucosaminidase 1 (HYAL-1) staining scores by tumor grade, stage, concomitant presence of carcinoma in situ (CIS), multifocality, gender, and age*

Parameter	HA			p value	HYAL-1			p value
	Mean	Median	95% CI		Mean	Median	95% CI	
Grade								
G1 (n = 51)	195.2 ± 66.42	200.0	176.5–213.9	G1 vs G2: $p > 0.05$ G1 vs G3: $p < 0.05$	170.8 ± 58.3	152	154.1–187.4	G1 vs G2: $p > 0.05$ G1 vs G3: $p < 0.001$
G2 (n = 89)	196.3 ± 62.81	190.0	183.1–209.6	G2 vs G3: $p < 0.05$	188.2 ± 57.0	197.5	175.4–201.1	G2 vs G3: $p < 0.01$
G3 (n = 38)	228.2 ± 65.62	230.0	206.6–249.8	–	231.4 ± 57.2	250.0	211.1–251.7	–
Stage								
Ta (n = 82)	210.4 ± 60.9	217.4	195.0–225.9	Ta vs T1: $p < 0.05$ Ta vs ≥T2: $p < 0.001$	162.3 ± 6.1	145.0	150.2–174.3	Ta vs T1: $p < 0.001$ Ta vs ≥T2: $p < 0.001$
T1 (n = 62)	180.5 ± 64.0	170.6	166.4–194.5	T1 vs ≥T2: $p > 0.05$	209.4 ± 7.7	215.0	193.9–225.0	T1 vs ≥T2: $p > 0.05$
≥T2 (n = 34)	242.8 ± 55.5	260.0	223.5–262.2	–	233.9 ± 49.8	240.0	215.1–252.8	–
Concomitant presence of CIS*								
Positive (n = 8)	231.5 ± 55.6	230.0	162.5–300.5	$p = 0.139$	248.0 ± 60.0	264.0	152.6–343.4	$p = 0.043$
Negative (n = 109)	187.7 ± 64.6	182.5	175.3–200.2		182.1 ± 59.8	183.0	170.1–194.2	
Gender								
Female (n = 132)	194.1 ± 63.2	175.1	180.5–207.8	$p = 0.21$	186.5 ± 66.1	167.0	156.5–216.6	$p = 0.97$
Male (n = 46)	175.1 ± 68.4	194.1	147.5–202.8		184.3 ± 59.9	193.0	171.0–197.6	
Multifocality*								
Positive (n = 66)	206.1 ± 67.80	206.1	184.4–227.8	$p = 0.036$	203.7 ± 60.4	211.5	184.3–223.0	$p = 0.014$
Negative (n = 51)	180.4 ± 61.39	180.4	165.9–195.0		172.4 ± 58.4	159.0	157.4–187.3	
Age								
<69 yr (n = 48)	197.1 ± 61.6	150	182.2–212	$p = 0.218$	171.2 ± 57.3	168	153.5–188.8	$p = 0.064$
≥69 yr (n = 69)	178 ± 68.4	201.3	156.9–199		194.8 ± 62	200	178.5–211.1	

CI = confidence interval.

* For patients with non-muscle-invasive bladder cancer only.

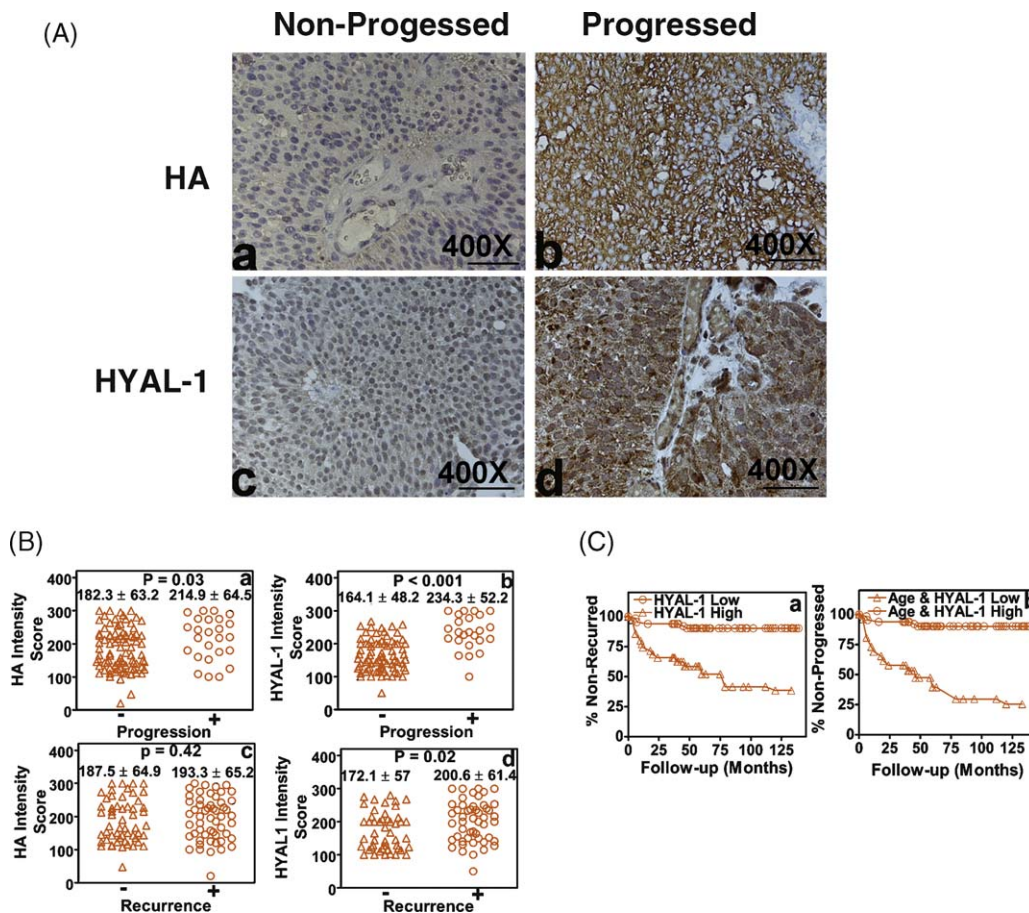


Fig. 1 – Hyaluronic acid (HA) and hyaluronoglucosaminidase 1 (HYAL-1) expression in bladder tumor tissue and its relation to tumor recurrence and progression to muscle invasion. (A) Localization of HA and HYAL-1 in bladder tissues: (a and b) HA; (c and d) HYAL-1. HA and HYAL-1 were localized in bladder tumor specimens from patients who did not (a and c) progress to muscle invasion and patients who had recurrence with muscle-invasive bladder tumor (b and d). Original magnification: $\times 400$. (B) HA and HYAL-1 scores. Scatter diagram of HA and HYAL-1 staining scores in tumor specimens, with respect to progression (a and b) or recurrence (c and d). The mean \pm SD scores for HA (a and c) and for HYAL-1 (b and d) staining intensity are indicated. Each bladder tumor specimen could receive a minimum possible score of zero and a maximum possible score of 300. (C) Kaplan-Meier plots. Kaplan-Meier plots were created to evaluate the effect of variables that were found to be independently associated with recurrence or progression to muscle invasion. (a) Stratification of the cohort to predict recurrence based on HYAL-1 staining intensity. HYAL-1 staining intensity significantly associated with tumor recurrence (as shown in Table 4). (b) The joint effect of age and HYAL-1 on progression to muscle invasion. Both parameters were found to independently associate with progression to muscle invasion (as shown in Table 4).

in the model (as individual variables), together with all of the clinical and pathologic parameters, only HYAL-1 was an independent prognostic predictor of tumor recurrence. Both HYAL-1 and age were significant parameters for predicting progression to muscle invasion (Table 4).

We next determined whether HYAL-1 staining inferences stratify patients with non-muscle-invasive BCa as those who recurred (or progressed to muscle invasion) versus those who did not [13,14]. The Kaplan-Meier plot shows that patients with high HYAL-1 staining scores (≥ 205 [the median HYAL-1 score]) in their tumor specimens recurred faster than those with low HYAL-1 staining (Fig. 1C, panel a; log-rank test: $\chi^2 = 13.12$; $p < 0.001$). Since both age and HYAL-1 staining were independent prognostic indicators in predicting progression to muscle invasion, we generated Kaplan-Meier plots by stratifying patients as low age (< 69 yr; median age: 69 yr) and low HYAL-1 (< 205) or

high age and high HYAL-1. As shown in panel b of Fig. 1C, patients with high age and high HYAL-1 progressed to muscle invasion faster (50% within 43 mo) than patients with low HYAL-1 staining (only 10% within 139 mo; log-rank test: $\chi^2 = 31.743$; $p < 0.001$).

To evaluate whether HA and, more importantly, HYAL-1 can accurately predict progression to muscle invasion and recurrence, we determined the sensitivity, specificity, and accuracy of the staining inferences. As shown in Table 5, HA staining had lower sensitivity, specificity, and accuracy than HYAL-1 staining to predict tumor recurrence and progression to muscle invasion, and the cross-validation analyses support these results.

Among the 111 patients for whom follow-up was available, 53 patients received intravesical therapy (ie, bacillus Calmette-Guérin [BCG] treatment) following TURBT. Of these 53 patients, 32 were responders; among the

Table 3 – Univariate analysis of pre- and postoperative parameters and hyaluronic acid (HA) and hyaluronoglucosaminidase 1 (HYAL-1) staining inferences*

Parameter	p value	χ^2	OR	95% CI
Progression				
HA	0.06	3.37	0.99	0.99–1.00
HYAL-1	<0.001**	15.73	1.02	1.01–1.03
T-stage	0.2	1.68	0.56	0.83–1.95
Grade	0.75	0.10	1.12	0.54–2.37
Multifocality	0.023**	5.19	1.66	1.07–2.6
CIS	0.37	0.8	1.52	0.54–3.8
Gender	0.59	0.28	1.14	0.67–1.85
Age	0.014**	6.04	0.95	0.91–0.99
Recurrence				
HA	0.41	0.69	0.99	0.99–1.00
HYAL-1	0.013**	6.19	1.01	1.00–1.02
T-stage	0.89	0.02	0.95	0.45–1.98
Grade	0.89	0.018	1.04	0.57–1.93
Multifocality	0.26	1.27	1.24	0.85–1.82
CIS	0.61	0.26	0.79	0.28–1.96
Gender	0.14	2.16	0.72	0.45–1.11
Age	0.45	0.56	0.99	0.96–1.02

CI = confidence interval; CIS = carcinoma in situ; OR = odds ratio.
 * Logistic regression single-parameter analysis was used to determine the association of preoperative (age, gender) and postoperative (grade, stage, CIS, multifocality) parameters and HA and HYAL-1 staining scores with progression to muscle invasion (stage T2 or higher) and tumor recurrence among patients with non-muscle-invasive bladder cancer. Because pT1 stage by definition invades subepithelial connective tissue, pTa and pT1 stages were evaluated separately.
 ** Statistically significant.

21 nonresponders, 17 experienced progression to muscle invasion. Analysis of the cohort that received intravesical therapy showed that the HYAL-1 staining score was higher in tumor specimens from patients who developed muscle-invasive disease (244.1 ± 46.1) when compared with those who did not (180.6 ± 60.2 ; $p = 0.0005$). In the multivariate analysis, HYAL-1 was the only independent prognostic

Table 4 – Multivariate analyses of pre- and postoperative parameters and hyaluronic acid (HA) and hyaluronoglucosaminidase 1 (HYAL-1) staining inferences to predict disease progression and tumor recurrence among patients with non-muscle-invasive bladder cancer*

Parameter	p value	χ^2	RR	95% CI
Recurrence				
HYAL-1	0.011	6.47	1.01**	1.00–1.01
Progression				
Age	0.049	3.99	1.06	1.00–1.12
HYAL-1	<0.001	13.47	1.02**	1.01–1.03

CI = confidence interval; CIS = carcinoma in situ; RR = risk ratio.
 * Cox proportional hazards analysis was performed by including preoperative (age, gender) and postoperative (tumor grade, stage, multifocality, concomitant presence of CIS) parameters and HA and HYAL-1 staining inferences to predict progression to muscle invasion and recurrence. Because pT1 stage by definition invades subepithelial connective tissue, pTa and pT1 stages were coded separately in the multivariate analysis. In both analyses, HA and HYAL-1 staining scores were included as continuous variables.
 ** Increase in risk per unit increase in HA or HYAL-1 staining score. In both models, only those parameters that reached statistical significance are shown.

indicator for predicting progression to muscle invasion ($\chi^2 = 10.4$; $p = 0.001$; 95% CI: 1.00–1.05).

4. Discussion

Prognostic markers that identify patients with non-muscle-invasive BCa who will develop muscle invasive disease could help physicians in choosing more aggressive treatment options (eg, early cystectomy). Palou et al, for example, showed that ezrin, a cytoskeletal protein involved in HA signaling, predicts response to BCG treatment [19]. In this study, we found that HYAL-1 expression is an independent predictor of progression to muscle invasion and tumor recurrence.

Table 5 – Determination of sensitivity, specificity, and accuracy of hyaluronic acid (HA) and hyaluronoglucosaminidase 1 (HYAL-1) staining inferences for predicting recurrence and progression to muscle invasion*

Parameter	HA		HYAL-1	
	Value	Cross-validation value, %	Value	Cross-validation value, %
Recurrence				
Sensitivity	61.8%	61.3 ± 3.8 95% CI: 51.7–80.3	57.6%	57.9 ± 3.1 95% CI: 50.2–71.7
Specificity	53.6%	54.5 ± 4.6 95% CI: 42.7–71.4	78%	78.3 ± 4.1 95% CI: 62–89.8
Accuracy	57.7%	57.6 ± 3.9 95% CI: 48.9–77.6	67.8%	66.5 ± 3.7 95% CI: 57.4–77.4
Progression to muscle invasion				
Sensitivity	76%	76.1 ± 4.3 95% CI: 65.4–90.6	78.3%	77.2 ± 4.1 95% CI: 63.8–92.4
Specificity	52.3%	52.5 ± 3.7 45.7 ± 61.8	75.3%	74.6 ± 2.8 95% CI: 64.5–91.7
Accuracy	64.2%	63.6 ± 3.2 95% CI: 54.6–72.9	76.8%	75.4 ± 2.9 95% CI: 65.2–92.4

CI = confidence interval.
 * The sensitivity, specificity, and accuracy values for HA and HYAL-1 staining were determined using the cut-off limits determined from the receiver operating characteristic curves and then cross-validated as previously described [18]. Data presented are mean plus or minus standard error of the mean and 95% CI.

The prognostic significance of HA expression in tumor cells and/or tumor-associated stroma may very well be organ specific [4,20–25]. In this study, HA staining score did not associate with progression to muscle invasion and tumor recurrence in both univariate and multivariate analyses. These findings are consistent with our observation that HA levels are only marginally higher in high-grade muscle-invasive tumors when compared with low-grade non-muscle-invasive tumors (Table 2) [15]. A marginal increase in HA levels in high-grade bladder-tumor specimens when compared with low-grade tumors may be due to elevated HYAL-1 levels in high-grade tumors. Increased HYAL-1 levels degrade tumor-associated HA, generating HA fragments. Such fragments have been detected in tumor tissues and in the urine of patients with high-grade BCa [26].

HYAL-1 expression was found to be an independent predictor of disease progression in breast and prostate carcinomas [13,14,27]. In this study, HYAL-1 expression was 77% accurate in identifying those patients with non-muscle-invasive BCa who later developed muscle-invasive BCa. Interestingly, HYAL-1 expression was higher in T1 tumors than in Ta tumors. For patients with T1G3 disease, it is often challenging to choose between cystectomy and bladder-sparing treatment that involves intravesical therapy. For such patients, early cystectomy may be a better treatment option if the tumor specimens have high HYAL-1 staining.

Our study included 34 patients with muscle-invasive disease, and of those, follow-up information was available for 31. Cox multivariate analysis that included preoperative (age, gender) and postoperative parameters (grade, stage, lymph node status) and HA and HYAL-1 staining inferences showed that HYAL-1 staining along with T-stage significantly associated with metastasis (HYAL-1, $p = 0.027$; stage, $p = 0.039$).

HYAL-1 promotes tumor invasion and metastasis. Inhibition of HYAL-1 expression in BCa and prostate cancer cells inhibits invasive activity in vitro and in xenografts [11,28]. In particular, inhibition of HYAL-1 expression in BCa xenografts inhibits skeletal muscle and lymphatic invasion [11]. Consistent with its role in tumor invasion and metastasis, HYAL-1 had lower sensitivity (57%) but comparable specificity (75%) to predict recurrence. This lower sensitivity was due to the poor sensitivity (approximately 40%) of HYAL-1 for predicting the recurrence of low-grade tumors.

5. Conclusions

As a whole, our study shows that, consistent with its functions in tumor invasion and metastasis, HYAL-1 has potential as an independent prognostic indicator for predicting progression to muscle invasion and metastasis. Our study, however, was a retrospective cohort study involving only two centers, and some of the patients had intravesical therapy following TURBT. Therefore, for HYAL-1 to be considered in treatment decision making [29] or to be included in BCa nomograms [30], a prospective multicenter study would have to be conducted.

Author contributions: Vinata Lokeshwar had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Kramer, Golshani, Merseburger, Lokeshwar.

Acquisition of data: Kramer, Golshani, Hennenlotter, Garcia.

Analysis and interpretation of data: Kramer, Golshani, Lokeshwar.

Drafting of the manuscript: Lokeshwar.

Critical revision of the manuscript for important intellectual content: Kuczyk, Stenzl, Soloway, Lokeshwar.

Statistical analysis: Duncan, Kramer, Lokeshwar.

Obtaining funding: Lokeshwar.

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Supervision: Lokeshwar.

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**Editorial Comment on: HYAL-1 Hyaluronidase:
A Potential Prognostic Indicator for Progression to
Muscle Invasion and Recurrence in Bladder Cancer**

Renzo Colombo

Department of Urology, “Vita-Salute” University,
Via Olgettina, 60, 20132 Milan, Italy
colombo.renzo@hsr.it

The risk assessment for recurrence and progression in non-muscle-invasive bladder cancer (NMIBC) patients represents one of the most challenging issues in urologic oncology. In these patients, the prognostic models are mainly based on clinical predictors (stage, grade, multifocality, associated carcinoma in situ, and failure after bacillus Calmette-Guérin [BCG] treatment), since none of the many molecular markers evaluated as potential prognostic factors has found its way into routine clinical practice.

In this original contribution, Kramer and coworkers [1] evaluated the potential role of HYAL-1 hyaluronidase as a prognostic indicator for both local recurrence and progression of NMIBC after transurethral resection (TUR).

The authors, who are very well-recognized authorities in the investigation of hyaluronic acid and HYAL-1, should be congratulated for the clarity of the text, the excellent

statistical analysis, and the cautious interpretation of their results.

HYAL-1-type hyaluronidase expression was already found to be an independent prognostic indicator of prostate cancer progression [2] and was significantly associated with subsequent cancer development in patients with benign breast lesions [3].

The enzymatically active HYAL-1 production by bladder cancer cells represents a well-documented molecular determinant of tumor growth, infiltration, and angiogenesis. For bladder cancer detection, HYAL-1 expression from urine specimens was already proved to be an accurate marker, providing a higher positive predictive value when compared with urinary cytology [4,5].

In this study, bladder cancer specimens were taken by TUR from NMIBC patients, and the expression of HYAL-1 in the specimens was graded for intensity and area of staining. HYAL-1 emerged as a significantly correlated predictor with the tendency to recur, from a statistical perspective. Additionally, based on a multivariate analysis including many clinical parameters, HYAL-1 appeared to be an independent prognostic indicator for progression.

A key issue is whether HYAL-1 expression evaluation actually provides any additional advantage over the well-known and more familiar clinical prognostic factors. Based on the model adopted for this study, the answer should be

yes. HYAL-1 was the only independent prognostic indicator of progression, thus providing value above tumor grade and stage.

Another key issue concerns the reproducibility of the methods used for the evaluation of HYAL-1 expression. Since the authors showed a significant correlation between the staining scores assigned by different readers and the IP image analysis software, we can argue that adequate image software could be used adequately in future clinical practice, thus overcoming the subjective interobserver variability.

A third issue concerns the cost-effectiveness analysis, but this factor was not addressed. The present study was only retrospective, it involved only two centers, and patients included those who were treated with adjuvant therapy as well as those who were not.

To confirm whether the biomarker in question may actually play a pivotal role in routine decision making, as admitted by the authors, prospective multicenter studies should be designed. Toward this end, clinical investigations could be focused on high-risk NMIBC patients after BCG failure. These patients require an accurate tool to correctly discriminate the candidates either for early

cystectomy or for salvage second-line conservative treatment.

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