

HYAL1 Hyaluronidase: A Molecular Determinant of Bladder Tumor Growth and Invasion

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Abstract

Hyaluronic acid and HYAL1-type hyaluronidase show high accuracy in detecting bladder cancer and evaluating its grade, respectively. Hyaluronic acid promotes tumor progression; however, the functions of hyaluronidase in cancer are largely unknown. In this study, we stably transfected HT1376 bladder cancer cells with HYAL1-sense (HYAL1-S), HYAL1-antisense (HYAL1-AS), or vector cDNA constructs. Whereas HYAL1-S transfectants produced 3-fold more HYAL1 than vector transfectants, HYAL1-AS transfectants showed ~90% reduction in HYAL1 production. HYAL1-AS transfectants grew four times slower than vector and HYAL1-S transfectants and were blocked in the G₂-M phase of the cell cycle. The expression of *cdc25c* and *cyclin B1* and *cdc2/p34*-associated H1 histone kinase activity also decreased in HYAL1-AS transfectants. HYAL1-S transfectants were 30% to 44% more invasive, and HYAL1-AS transfectants were ~50% less invasive than the vector transfectants *in vitro*. In xenografts, there was a 4- to 5-fold delay in the generation of palpable HYAL1-AS tumors, and the weight of HYAL1-AS tumors was 9- to 17-fold less than vector and HYAL1-S tumors, respectively ($P < 0.001$). Whereas HYAL1-S and vector tumors infiltrated skeletal muscle and blood vessels, HYAL1-AS tumors resembled benign neoplasia. HYAL1-S and vector tumors expressed significantly higher amounts of HYAL1 (in tumor cells) and hyaluronic acid (in tumor-associated stroma) than HYAL1-AS tumors. Microvessel density in HYAL1-S tumors was 3.8- and 9.5-fold higher than that in vector and HYAL1-AS tumors, respectively. These results show that HYAL1 expression in bladder cancer cells regulates tumor growth and progression and therefore serves as a marker for high-grade bladder cancer. (Cancer Res 2005; 65(6): 2243–50)

Introduction

Bladder tumors, particularly transitional cell carcinomas, show heterogeneity in their ability to invade and metastasize (1–4). For example, low-grade bladder tumors rarely progress, whereas about two-thirds of high-grade tumors are detected at stages $\geq T1$ (i.e., invading lamina propria and beyond; ref. 5). Muscle invasion by a bladder tumor indicates poor prognosis, as 50% of patients develop metastases within 2 years and 60% die within 5 years regardless of treatment (6). Certain molecular markers, such as hyaluronidase, have been identified as highly sensitive and specific markers for detecting high-grade (i.e., grade 2 and 3) bladder cancer (7, 8).

However, the functions of hyaluronidase (if any) in bladder tumor growth and/or invasion are unknown.

Hyaluronic acid is the substrate of hyaluronidase. It is a glycosaminoglycan made up of repeating disaccharide units, D-glucuronic acid, and N-acetyl-D-glucosamine (9). Hyaluronic acid is normally present in tissues and body fluids. Hyaluronic acid keeps tissues hydrated in an osmotically balanced environment (10). It also regulates cell proliferation, migration, and adhesion by interacting with cell surface hyaluronic acid receptors, such as CD44 (11). Concentrations of hyaluronic acid are elevated in cancers of the breast, colon, prostate, bladder, etc. (8, 12–18). In tumor tissues, elevated hyaluronic acid is mostly localized to tumor stroma; however, in some tumors, including bladder cancer, it is also expressed in tumor cells (12–18). We have shown previously that hyaluronic acid levels are elevated in the urine of bladder cancer patients regardless of the tumor grade (8, 19). Thus, the measurement of urinary hyaluronic acid levels (hyaluronic acid test) has 83% sensitivity and 90% specificity for detecting bladder cancer (8). In tumor tissues, hyaluronic acid swells on hydration and opens up spaces for tumor cell migration, and tumor cells migrate on hyaluronic acid-rich matrix by interacting with hyaluronic acid receptors (10, 11). A hyaluronic acid coat around tumor cells causes a partial loss of contact-mediated growth and migration and offers protection against immune surveillance (20–23). Small fragments of hyaluronic acid (i.e., 3–25 disaccharide units) are angiogenic (24, 25). We have isolated previously such small fragments from the urine of grade 2/3 bladder cancer patients, high-grade prostate cancer tissues, and saliva of head and neck cancer patients (18, 19, 26).

Small fragments of hyaluronic acid are generated by limited digestion of the hyaluronic acid polymer with hyaluronidase. Hyaluronidase levels have been shown to be elevated in cancers of the prostate, bladder, and head and neck cancer and in breast tumors and malignant glioma (7, 19, 26–32). For example, we have shown that urinary hyaluronidase levels measured using an ELISA-like assay (hyaluronidase test) is a highly sensitive (81% sensitivity) and specific (83.8% specificity) marker for detecting grade 2 and 3 bladder tumors (7, 8). Elevated hyaluronidase levels also seem to be a sensitive marker for detecting head and neck cancer (26). We partially purified and characterized the first tumor-derived hyaluronidase from the urine of high-grade bladder cancer patients and showed its identity to HYAL1 (33). Subsequently, we showed HYAL1 expression in several invasive bladder, prostate, and head and neck tumor cell lines (18, 26, 33). For example, we analyzed HYAL1 expression and hyaluronidase secretion in 11 bladder cancer cell lines (33). Among these, HT1376 cells secrete the highest amount of hyaluronidase activity (32 ± 2.4 mU/mg protein) in their conditioned medium, and HYAL1 is the major hyaluronidase expressed in these cells. We showed recently that HYAL1 expression in radical prostatectomy specimens is an independent predictor of biochemical recurrence in prostate cancer patients

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(27, 34). In tumor cells, the expression of enzymatically active HYAL1 seems to be regulated by alternative mRNA splicing (35–37).

Jacobson et al. reported recently that overexpression of HYAL1 in a rat colon cancer line suppresses tumor growth in xenografts (38). Contrarily, Victor et al. showed that passage of a human breast cancer line CAL 51 from the primary state to metastatic stage increases hyaluronidase production (39). Expression of HYAL1 in a prostate cancer line that produces little hyaluronidase did not affect tumor growth but caused a slight increase in lung metastasis (40). Thus, the functional significance of HYAL1 expression in human tumor cells, which normally express HYAL1, is still unknown.

At present, it is also unknown whether HYAL1 is only a marker for more aggressive bladder cancer or it also functions as one of the molecular determinants that control bladder tumor growth and invasion. In this study, we examined the function of HYAL1 in bladder tumor growth, invasion, and angiogenesis using HYAL1-antisense (HYAL1-AS) transfection to block HYAL1 expression and HYAL1-sense (HYAL1-S) cDNA transfection to overexpress HYAL1.

Materials and Methods

Construction of HYAL1-S and HYAL1-AS cDNA Constructs. HYAL1 cDNA containing the entire coding region was amplified by reverse transcription-PCR analysis and cloned into a eukaryotic expression vector, pcDNA3.1/v5-His TOPO, using a TOPO-TA cloning kit (Invitrogen, Carlsbad, CA; ref. 35). The TOPO-TA cloning allowed bidirectional cloning of the HYAL1 cDNA insert with respect to the cytomegalovirus promoter (i.e., HYAL1-S and HYAL1-AS cDNA constructs). HYAL1-S, HYAL1-AS, and vector cDNA constructs were used for transfection studies.

Generation of HYAL1 Transfectants. HT1376, a transitional cell carcinoma of the bladder cell line, was cultured in RPMI 1640 containing 10% fetal bovine serum and gentamicin (growth medium). HT1376 cells (2×10^5 per 6 cm dish) were transfected with 5 μ g of vector, HYAL1-S, or HYAL1-AS cDNA constructs using the Superfect transfection reagent (Qiagen, Valencia, CA). The transfectants were selected in growth medium containing 200 μ g/mL geneticin (Invitrogen).

Analysis of Hyaluronidase Activity by Hyaluronidase ELISA-Like Assay. Hyaluronidase activity in serum-free conditioned medium of transfectants (1×10^6 cells) was assayed using the hyaluronidase ELISA-like assay (7, 8, 33). Hyaluronidase activity (mU/mL) was normalized to total protein concentration (mg/mL) and was expressed as mU/mg.

Substrate (Hyaluronic Acid)-Gel Assay. Conditioned media from HT1376 transfectants (secreted by 5×10^4 cells, ~ 10 μ g total protein) were separated on a substrate (hyaluronic acid)-gel. Following incubation in a hyaluronidase assay buffer, the gel was stained and destained to visualize active hyaluronidase species (7, 18).

Immunoblot Analysis. Conditioned media from the transfectant clones (5×10^4 cells, ~ 10 μ g total protein) were immunoblotted using an anti-HYAL1 peptide IgG (i.e., anti-HYAL1 IgG) as described previously (18, 33). Cell lysates (4×10^4 cells per transfectant) were subjected to immunoblot analysis using the following primary antibodies: 1 μ g/mL mouse anti-cyclin B1 IgG (clone GNS1, Neomarkers, Labvision, Fremont, CA), 1 μ g/mL mouse anti-cdc2/p34 IgG (clone A27.1.1 with POH-1), or 0.2 μ g/mL rabbit anti-cdc25c IgG (C-20, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Protein loading was evaluated by reprobing the blots with mouse anti-actin IgG (Neomarkers).

Cell Proliferation Assay. HT1376 transfectants (2×10^4 cells per well) were plated on 24-well culture plates in growth medium with geneticin. Every 24 hours for a total period of 5 days (0–120 hours), cells were trypsinized and counted following trypan blue staining. Counts were obtained from triplicate wells in two independent experiments.

Cell Cycle Analysis. HT1376 transfectant cultures (60% confluence) were lysed in a propidium iodide dye solution (0.1% sodium citrate, 0.4% NP40, and 25 μ g/mL propidium iodide) and analyzed in an EPICS XL flow cytometer equipped with a long pass red filter, FL3 (630 nm). The FL3 histograms were analyzed for estimating cell cycle phase distribution by

Modfit Easy (Lite) program (Veritas Software, Mountainview, CA; ref. 41). All samples were assayed in duplicates in two independent experiments.

Immunoprecipitation and Kinase Assay. HT1376 transfectant cells (1×10^6) were solubilized in a lysis buffer and immunoprecipitated using 2 μ g/mL mouse anti-cdc2/p34 IgG and protein A agarose (Sigma-Aldrich, St. Louis, MO). In control samples, the primary antibody was excluded. The immunoprecipitates were incubated with histone H1 in a hot kinase solution (2.5 μ g H1 histone, 5 μ mol/L ATP, 5 μ Ci [γ - 32 P]ATP in kinase buffer) at 37°C for 30 minutes. Histone H1 was analyzed by 12% SDS-PAGE and autoradiography (42).

Analysis of Apoptosis. Forty-eight-hour cultures of transfectants (10^5 cells per 24-well plate) were lysed and the cell lysates were tested for free nucleosome release using the Cell Death ELISA kit (Roche Diagnostics, Pleasanton, CA). All samples were assayed in triplicates in two independent experiments.

Matrigel Invasion Assay. The membranes in 12-well Transwell plates (Corning-Costar, Corning, NY) were coated with Matrigel (100 μ g/cm 2) or Matrigel with hyaluronic acid (50 μ g/mL) in serum-free medium. HT1376

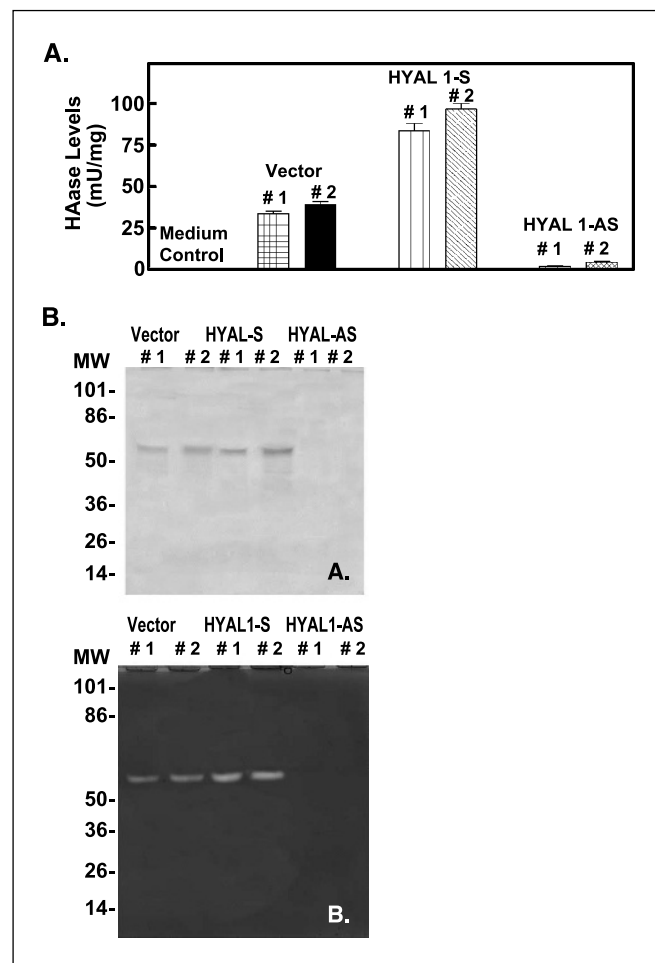


Figure 1. Analysis of HYAL1 expression in HT1376 transfectants. **A**, measurement of hyaluronidase (HAase) activity. Columns, mean hyaluronidase activity (mU/mg) from three separate experiments; bars, SE. **B**, immunoblot analysis using anti-HYAL1 IgG. Conditioned media (10 μ g protein; conditioned medium of 5×10^4 cells) from each transfectant clone were subjected to anti-HYAL1 IgG immunoblotting as described in Materials and Methods. **C**, substrate (hyaluronic acid)-gel assay. Conditioned media (10 μ g protein; conditioned medium of 5×10^4 cells) from each transfectant clone were analyzed by substrate (hyaluronic acid)-gel. In the absence of a specific and well-accepted protein loading control for secreted protein, we used cell number and total protein for normalizing the total amount of conditioned medium for each transfectant, for HYAL1 immunoblot analysis, and for substrate (hyaluronic acid)-gel assay.

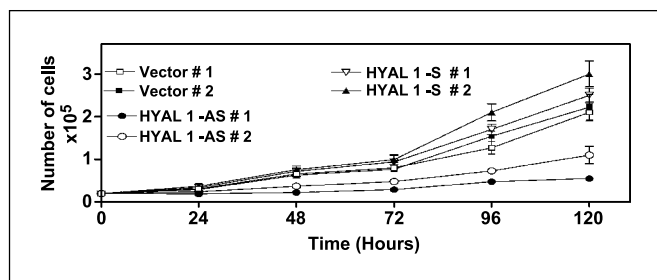


Figure 2. Determination of the proliferation rate of HT1376 transfectants. Points, mean cell count from triplicate measurements in two independent measurements; bars, SD.

transfectants (3×10^5 cells per well) were plated on the upper chamber in serum-free medium. The bottom chamber contained growth medium with geneticin. Following 48-hour incubation, invasion of cells through Matrigel into the bottom chamber was quantified using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (41). Invasion of cells was calculated as (cells in the bottom chamber) / (cells in upper and bottom chambers) \times 100. Invasion by vector transfectants was normalized as 100% (control). Invasive activity of each clone was determined in triplicates in two independent experiments.

Pericellular Matrix (Coat) Assay. Pericellular matrices (coats) around transfectants were visualized using a particle exclusion assay involving formaldehyde fixed human erythrocytes as described previously (43, 44). Cells were counted in 10 fields (127-155 cells per transfectant) per dish and in two dishes per transfectant. Cells, which showed a phase bright region around the entire periphery, with an average width greater than or equal to the diameter of one erythrocyte, were counted as having a pericellular matrix. Results were expressed as percentage \pm SD of cells with pericellular matrix.

Tumor Xenografts. HT1376 transfectants (2×10^6 or 4×10^6 cells per animal, 10 animals per group) were s.c. implanted on the right dorsal flank of 6-week-old female athymic mice. After tumors became palpable, tumor size was measured twice weekly and tumor volume was calculated assuming an ellipsoid shape. At day 30, all 10 animals from vector and HYAL1-S transfectants and 5 animals from HYAL1-AS groups were euthanized. The remaining 5 mice in HYAL1-AS group were euthanized at day 60. Difference in tumor growth rate (i.e., generation of palpable tumors) and tumor weight at day 30 were statistically evaluated by Tukey-Kramer multiple comparisons test. The experiment was repeated once. Tumor histology was done at Charles River Laboratories (Wilmington, MA).

Immunohistochemistry: Hyaluronic Acid and HYAL1 Localization. Hyaluronic acid and HYAL1 were localized in tumor xenograft specimens by immunohistochemistry using a biotinylated bovine nasal cartilage hyaluronic acid-binding protein and the rabbit anti-HYAL1 IgG as described previously (17, 18, 27, 34). The hyaluronic acid- and HYAL1-stained slides were graded with respect to staining intensity 0 or 1+ (weak), 2+ (moderate), and 3+ (high) staining. All the authors read the slides independently.

Microvessel Density Determination. To visualize microvessels, slides containing tumor specimens were incubated with 3.1 μ g/mL rat anti-mouse CD34 IgG (BD PharMingen, San Diego, CA) at 4°C for 18 hours. The slides were then sequentially incubated with a biotinylated rabbit anti-rat IgG, an avidin-biotin peroxidase conjugate solution (anti-rat ABC kit, Vector Laboratories, Burlingame, CA), and 3,3'-diaminobenzidine substrate solution (DAKO Laboratories, Carpinteria, CA). The slides were counterstained with hematoxylin and the microvessel density (MVD) was determined by counting the anti-CD34-stained microvessels (34). MVD was determined by two readers independently by choosing the hotspots and counting the microvessels. MVD was expressed as mean \pm SD.

Results

Analysis of HYAL1 Expression in HT1376 Transfectants. Because HT1376 cells secrete the highest amount of hyaluronidase activity among the 11 bladder cancer cell lines (33), we chose HT1376 cell line to generate HYAL1-S (hyaluronidase-overproducing) and HYAL1-AS (hyaluronidase-non-producing) stable transfectants. We analyzed 25 to 30 stable clones of each transfectant type for analysis, and data on two clones from each category are presented. As shown in Fig. 1A, hyaluronidase activity (mU/mg) secreted by HYAL1-S (1: 83.5 ± 4.5 and 2: 94.5 ± 3.5) transfectants is \sim 2.5-fold when compared with the vector (1: 33.5 ± 1.5 and 2: 38.5 ± 2.5) transfectants. There is $>90\%$ reduction in the amount of hyaluronidase secreted by HYAL1-AS (1: 2.0 ± 0.5 and 2: 4.3 ± 0.4) transfectants when compared with vector transfectants (Fig. 1A). The amount of hyaluronidase activity secreted by HYAL1-AS transfectants is similar to that secreted by noninvasive bladder cancer cell lines, such as RT4 (33). As shown in Fig. 1B, a \sim 60-kDa HYAL1 protein is detected in the conditioned medium of vector (1 and 2) and HYAL1-S (1 and 2) transfectants. However, this protein is not detected in the conditioned medium of HYAL1-AS (1 and 2) transfectant conditioned medium. The substrate (hyaluronic acid)-gel analysis confirms the presence of a \sim 60-kDa active hyaluronidase species in the conditioned medium of both vector and HYAL1-S transfectants. As expected, the active hyaluronidase species is not detected in the conditioned medium of HYAL1-AS transfectants (Fig. 1C).

Effect of HYAL1 Expression on Cell Proliferation, Cell Cycle, and Apoptosis. The growth rate of vector and HYAL1-S transfectants is comparable (Fig. 2). The doubling time of both vector transfectant clones is \sim 30 hours and that of HYAL1-S 1 and HYAL1-S 2 transfectants is 26 and 24 hours, respectively. HYAL1-AS transfectants, however, grew \sim 4 times slower when compared with vector and HYAL1-S transfectants (doubling time, 1: 96 hours and 2: 80 hours; Fig. 2).

As shown in Table 1, HYAL1 expression seems to affect the G_2 -M phase of the cell cycle. There is a 200% and $\geq 500\%$ increase in the

Table 1. Cell cycle analysis of HT1376 transfectants: HYAL1-S, HYAL1-AS, and vector transfectant clones subjected to flow cytometry for cell cycle analysis

| Phase | Vector 1 (%) | Vector 2 (%) | HYAL1-S 1 (%) | HYAL1-S 2 (%) | HYAL1-AS 1 (%) | HYAL1-AS 2 (%) |
|---------------|--------------|--------------|---------------|---------------|----------------|----------------|
| G_0 - G_1 | 56.7 | 55.8 | 55.2 | 54.2 | 55.9 | 57.1 |
| S | 37.7 | 38.4 | 42.4 | 44.3 | 31.4 | 32.7 |
| G_2 -M | 5.6 | 5.8 | 2.4 | 1.5 | 12.7 | 10.2 |

NOTE: Percentages of cells in G_0 - G_1 , S, and G_2 -M phases of the cell cycle are shown. Average of duplicates from two independent experiments. SD \leq 5%.

number of HYAL1-AS transfectants in G₂-M phase when compared with the vector and HYAL1-S transfectants, respectively (<0.001; Tukey-Kramer multiple comparisons test). Correspondingly, the percentage of HYAL1-AS cells in S phase decreases when compared with vector and HYAL1-S cells. HYAL1 expression does not affect the G₀-G₁ phase (Table 1).

We next analyzed the expression of G₂-M regulators (i.e., cdc25c, cyclin B1, and cdc2/p34 proteins) in HT1376 transfectant clones. There is a 3- to 4-fold decrease in the expression of cdc25c and cyclin B1 in HYAL1-AS transfectant clones when compared with that in vector and HYAL1-S transfectants, respectively (Fig. 3A). The expression of cdc2/p34 in HYAL1-AS transfectants does not change in HYAL1-AS transfectants when compared with the vector transfectants but decreases ~1.5-fold when compared with HYAL1-S transfectants (Fig. 3A). There is also a ~2- and ~4-fold decrease in the cdc2/p34-associated H1 histone kinase activity in HYAL1-AS transfectant clones when compared with the vector and HYAL1-S transfectants, respectively (Fig. 3B). These results show that HYAL1-AS transfectants are arrested in the G₂-M phase of the cell cycle.

We also determined whether HYAL1 expression affects apoptosis. As shown in Table 2, there are no significant differences in apoptosis among vector, HYAL1-S, and HYAL1-AS clones. This was further confirmed by using Annexin V binding to study outward translocation of plasma membrane phosphatidylserine among HYAL1 transfectants. No differences in phosphatidylserine translocation were observed among vector (1 and 2), HYAL1-S (1 and 2), and HYAL1-AS (1 and 2) transfectants. These results show that inhibition of HYAL1 expression affects cell cycle progression but not apoptosis.

Effect of HYAL1 Expression on Invasion. The invasive activity of vector transfectant clones ($28.8 \pm 2.3\%$) was normalized as 100%. As shown in Fig. 4A, HYAL1-AS transfectants 1 and 2 are 40% and 45% less invasive than the vector transfectant clones. Contrarily, HYAL1-S transfectants are 140% (1) and 159% (2) more invasive than vector transfectants. Incorporation of hyaluronic acid in Matrigel does not influence the invasive properties of various HT1376 transfectants (Fig. 4B). These results show that blocking of

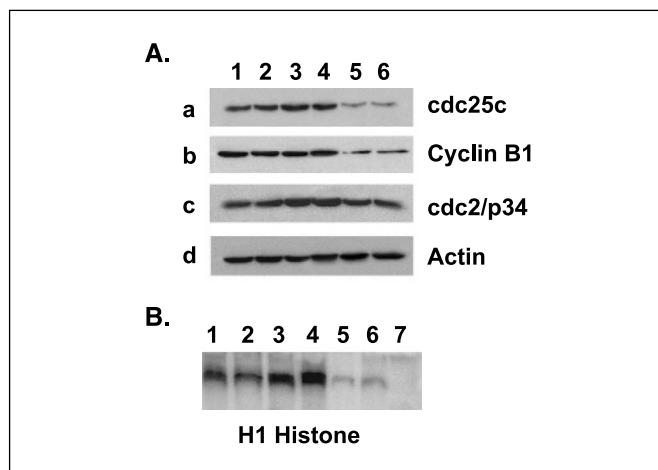


Figure 3. Analysis of G₂-M cell cycle regulators in HT1376 transfectants. A, cell lysates of HT1376 transfectants were analyzed by immunoblotting using anti-cdc25c (a), anti-cyclin B1 (b), anti-cdc2/p34 (c), and β -actin (d) antibodies. Lanes 1 and 2, vector clones 1 and 2; lanes 3 and 4, HYAL1-S clones 1 and 2; lanes 5 and 6, HYAL1-AS clones 1 and 2. B, H1 histone kinase-associated activity of cdc2/p34 was measured as described in Materials and Methods. Lanes 1 and 2, vector clones 1 and 2; lanes 3 and 4, HYAL1-S clones 1 and 2; lanes 5 and 6, HYAL1-AS clones 1 and 2; lane 7, negative control.

Table 2. Analysis of apoptosis in HT1376 transfectants

| Transfectant | Free nucleosome A ₄₀₅ | % of Control |
|--------------------|----------------------------------|--------------|
| Vector 1 (control) | 1.8 ± 0.05 | 100 |
| Vector 2 | 1.5 ± 0.25 | 83.3 |
| HYAL1-S 1 | 1.79 ± 0.21 | 99.4 |
| HYAL1-S 2 | 1.91 ± 0.37 | 106 |
| HYAL1-AS 1 | 1.46 ± 0.16 | 81.1 |
| HYAL1-AS 2 | 1.83 ± 0.2 | 102 |

NOTE: Apoptotic activity in vector, HYAL1-S, and HYAL1-AS transfectant clones was determined. Apoptotic activity in vector 1 clone was considered as 100% (control). Average \pm SE A₄₀₅ from duplicate measurements in two independent experiments.

HYAL1 expression significantly reduces the invasive activity of bladder cancer cells.

Effect of HYAL1 Expression on Hyaluronic Acid-Dependent Pericellular Matrix Formation. As shown in Fig. 5, the vector and HYAL1-S clones do not exhibit pericellular matrices, as the erythrocytes closely abut the surface of each cell and in some cases cover the cells. However, HYAL1-AS cells exhibit a pericellular matrix, as the erythrocytes do not penetrate the matrix and a clear coat surrounds the cells. The percentage of cells with pericellular matrix was elevated in HYAL1-AS transfectants (1: 85.6 ± 15.9 and 2: 86.7 ± 14.3) when compared with the vector (1: 56.3 ± 16.7 and 2: 47.8 ± 15.3) and HYAL1-S (1: 21.7 ± 16.25 and 2: 19.61 ± 14.83) transfectants. The differences among HYAL1-AS and vector, HYAL1-AS and HYAL1-S, and HYAL1-S and vector were statistically significant ($P < 0.001$). Thus, hyaluronic acid is an important component of the pericellular matrix that surrounds bladder cancer cells.

Effect of HYAL1 Expression on Tumor Xenografts. As shown in Fig. 5A, when injected at 2×10^6 cells per site density, there was a 4- to 5-fold delay in the generation of palpable tumors in

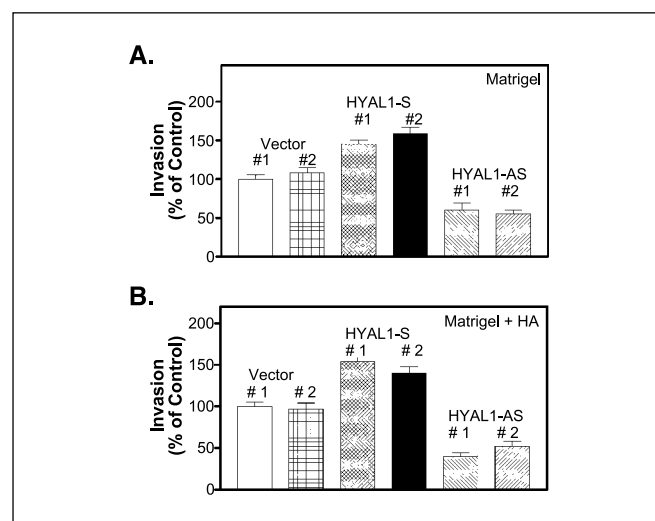


Figure 4. Determination of the invasive activity of HT1376 transfectants *in vitro*. Invasive activity was tested in Matrigel alone (A) or Matrigel + hyaluronic acid (B). Invasive activity of vector transfectant clone 1 (control) was considered as 100%. Columns, mean from triplicate determinations in two independent experiments; bars, SD.

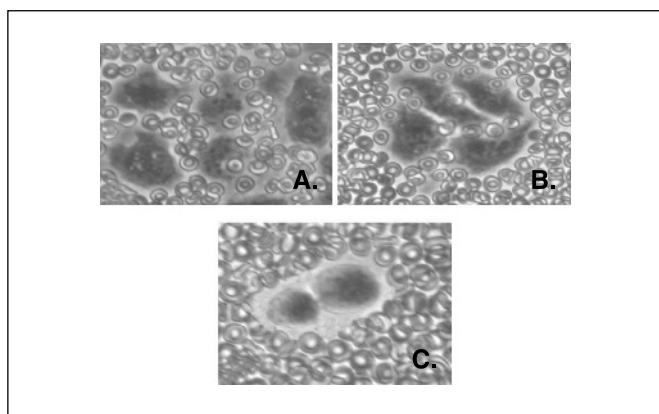


Figure 5. Examination of pericellular matrix in HT1376 transfectants: pericellular matrices surrounding various HT1376 transfectants were visualized using the particle exclusion assay. Human erythrocytes surrounding tumor cells: (A) vector 2, (B) HYAL1-S 2, and (C) HYAL1-AS 2.

animals injected with HYAL1-AS transfectant (40 ± 3 days) when compared with animals injected with HYAL1-S (7 days) and vector (10 ± 2 days) transfectants, respectively ($P < 0.001$). The weight of HYAL1-AS transfectant tumors is 9.1-fold less than that of vector tumors and 17.3-fold less than that of HYAL1-S tumors, respectively (Fig. 5B; $P < 0.001$). The 1.9-fold increase in the weight of HYAL1-S tumors compared with vector tumors is also statistically significant ($P < 0.05$). The picture of two representative tumors from each transfectant group shows that HYAL1-AS tumors are indeed much smaller than the vector and HYAL1-S tumors (Fig. 5C).

We also injected various transfectants at 4×10^6 cells per site. As shown in Fig. 5D, the weight of HYAL1-AS tumors is still 10- and 17.7-fold less when compared with the weights of vector and HYAL1-S tumors, respectively ($P < 0.001$). Therefore, blocking HYAL1 expression decreases tumor growth regardless of the initial tumor inoculum.

Tumor histology report provided by Charles River Laboratories stated that vector and HYAL1-S tumors grew by infiltrating surrounding tissues, including skeletal muscle. The tumors also contained numerous blood vessels. On the contrary, HYAL1-AS tumor growth was described as resembling benign neoplasm. Figure 6 shows the photomicrographs of representative tumor histology corresponding to vector, HYAL1-S, and HYAL1-AS tumors. As shown in Fig. 6A, clusters of tumor cells have infiltrated the skeletal muscle, and two blood vessels are present at the periphery. At higher magnification, tumor cells are adjacent to skeletal muscle fibers. A blood vessel is also present close to tumor cells at the lower left (Fig. 6D). In the HYAL1-S tumor specimen, the skeletal muscle fibers are entrapped in tumor cells, as are the blood vessels (Fig. 6B). At higher magnification, tumor cells are present at the edge of a blood vessel, indicating infiltration. The tumor cells are surrounding the skeletal muscle fibers and also show a high number of mitotic figures (Fig. 6E). As shown in Fig. 6C, the HYAL1-AS tumor has a discrete margin and there is no evidence of infiltration into skeletal muscle. In addition, no large vessels are present in the specimen. At higher magnification, the specimen does not contain any skeletal muscle fibers and only a single capillary is present in the center (Fig. 6F). These results show that HYAL1 expression influences the invasive phenotype of bladder tumor cells *in vivo*.

Expression of Hyaluronic Acid, HYAL1, and Microvessel Density. To determine whether tumor cells in vector, HYAL1-S, and

HYAL1-AS tumor specimens retain their phenotype, we localized HYAL1 and hyaluronic acid in tumor xenografts. Tumor cells in the vector specimen show moderate expression of HYAL1 (2+ staining intensity), whereas tumor cells in HYAL1-S specimen show high level of HYAL1 expression (3+ staining intensity; Fig. 7a-c). None to very little HYAL1 staining is observed in HYAL1-AS tumor specimen (0 to 1+ staining intensity). In the vector tumor specimen, there is moderate (2+ staining intensity) hyaluronic acid expression in the tumor-associated stroma, but tumor cells are negative for hyaluronic acid expression (Fig. 7A, d). There is high level of hyaluronic acid expression in tumor-associated stroma in HYAL1-S tumor specimen (Fig. 7A, e). Interestingly, very low hyaluronic acid expression is observed in the stromal compartment in HYAL1-AS tumor specimens (Fig. 7A, f).

As shown in Fig. 7B, MVD in HYAL1-S tumor specimens (127.2 ± 29.23 ; range, 97-196) is 3.8-fold higher than that in vector tumor specimens (33.86 ± 5.55 ; range, 27-45) and 9.5-fold higher than that in HYAL1-AS specimens (13.43 ± 5.09 ; range, 6-22; $P < 0.001$; Tukey-Kramer multiple comparisons test; Fig. 8).

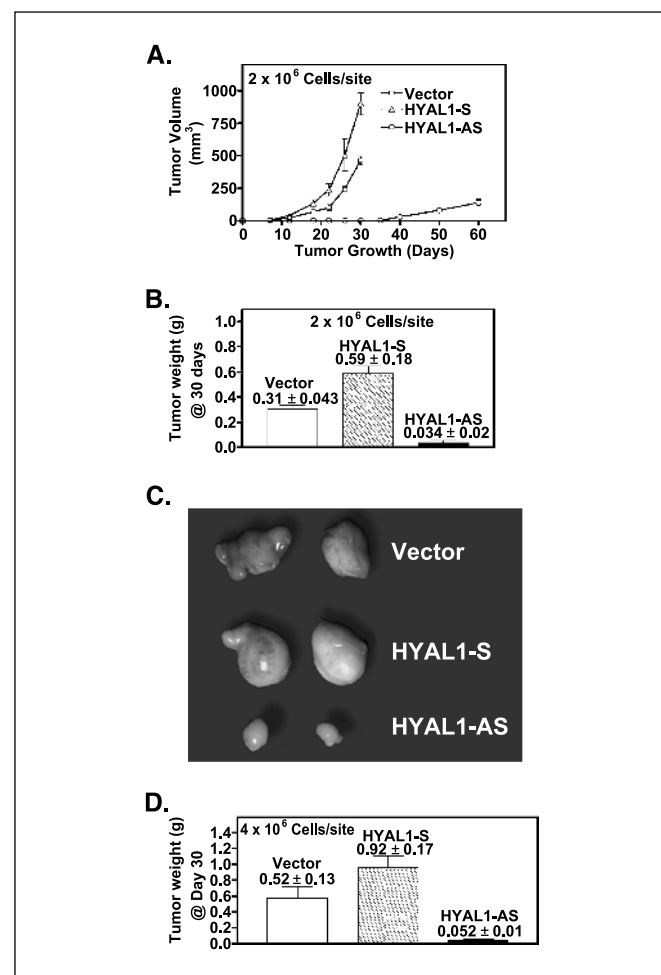


Figure 6. Examination of the growth of HT1376 transfectant tumors in xenografts. Vector 2, HYAL1-S 2, and HYAL1-AS 2 transfectant clones were injected s.c. in athymic mice (10 animals per group) at 2×10^6 cells per site density. A, points, mean tumor volume; bars, SD. B, columns, mean tumor weight (g); bars, SD. C, two representative tumors from each group (i.e., vector, HYAL1-S, and HYAL1-AS) taken at necropsy. D, vector 2, HYAL1-S 2, and HYAL1-AS 2 transfectant clones were injected s.c. in athymic mice at 4×10^6 cells per site density. Columns, mean tumor weight (g); bars, SD.

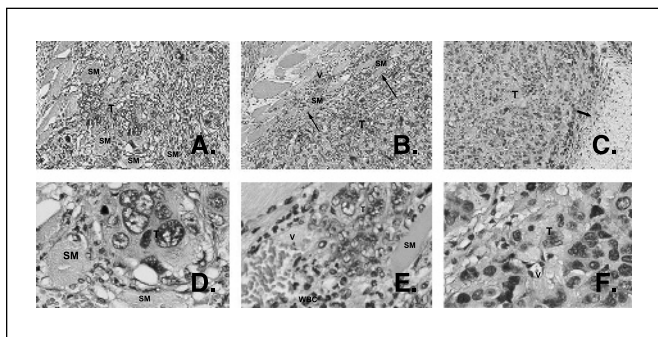


Figure 7. Examination of histology of transfectant tumors. Charles River Laboratories provided the tumor histology pictures (A-F). A-C, magnification, $\times 100$. D-F, magnification, $\times 400$. SM, skeletal muscle fiber; T, tumor or tumor cells; V, blood vessel; WBC, leukocytes. Arrows, entrapment of skeletal muscle fibers by tumor.

Discussion

Our results show that blocking HYAL1 expression in a bladder cancer line results in a 4-fold decrease in cell growth rate, suggesting that HYAL1 expression by tumor cells is required for cell proliferation. The proliferation rate of HYAL1-S transfectants, however, is not significantly higher than that of the vector transfectants. Because HYAL1-S transfectants secrete only ~ 2.5 -fold more HYAL1 than vector transfectants, moderate overexpression of HYAL1 in a bladder cancer cell line that already produces significantly higher amounts of HYAL1 (30 mU/mg) may not appreciably alter the cell proliferation rate. Our results, which show that blocking of HYAL1 expression induces cell cycle arrest, are consistent with a report that HYAL1 expression in an oral squamous cell carcinoma line induces S-phase entry (45). Based on the analysis of cell cycle and G₂-M regulators, HYAL1 expression very likely affects cell proliferation by regulating cell cycle.

Our finding that HYAL1-AS transfectants are $\sim 50\%$ less invasive than vector transfectants and that HYAL1-S transfectants are more invasive than vector transfectants are consistent with our previous observations that HYAL1 levels are elevated in high-grade bladder tumor tissues and in patients' urine (8, 17). Most high-grade tumors given sufficient time will invade bladder muscle and metastasize and therefore present with poor prognosis (1-4).

In addition to the effect of HYAL1 on tumor growth, its effects on tumor infiltration into skeletal muscle are interesting. HYAL1-S and vector tumors infiltrated skeletal muscle. HYAL1-AS tumors were benign and did not invade the muscle. Muscle invasion by bladder tumor is independent of tumor volume and is ominous, as 60% of patients have distant metastasis within 2 years and 50% die within 5 years (1-6). The observations of this study suggest that HYAL1 plays a role in promoting the invasive potential of bladder tumor cells. It may also explain why urinary hyaluronidase levels serve as an accurate marker for detecting high-grade bladder cancer but are not elevated in patients with low-grade tumors (1-4). HYAL1 is also an independent prognostic indicator for predicting biochemical recurrence in prostate cancer and increases metastatic potential of a prostate cancer line (29, 34, 40). Taken together, HYAL1 seems to function in bladder tumor growth and invasion.

In tumor xenografts, hyaluronic acid was exclusively localized in tumor-associated stroma, whereas HYAL1 was expressed by tumor cells. We have shown previously such dichotomy of hyaluronic acid and HYAL1 expression in prostate cancer (18). There is also a synergy

between hyaluronic acid and HYAL1 expression. For example, there was considerably less hyaluronic acid in the stroma in HYAL1-AS tumors than there was in vector and HYAL1-S tumors. These observations are consistent with the expression of hyaluronic acid and HYAL1 in human bladder tumors. For example, there is low expression of hyaluronic acid and HYAL1 in grade 1 tumors when compared with grade 2 and 3 tumors, respectively (17). The synergy between stromal hyaluronic acid and tumor cell-HYAL1 expression suggests that one or both of these molecules may influence each other's synthesis in the tumor microenvironment.

One of the well-studied functions of the hyaluronic acid and hyaluronidase system is the generation of angiogenic hyaluronic acid fragments (24, 25). These angiogenic hyaluronic acid fragments have been shown to induce endothelial cell proliferation, migration, and adhesion (46-48). The secretion of hyaluronidase by tumor cells has been shown to induce angiogenesis (32), whereas

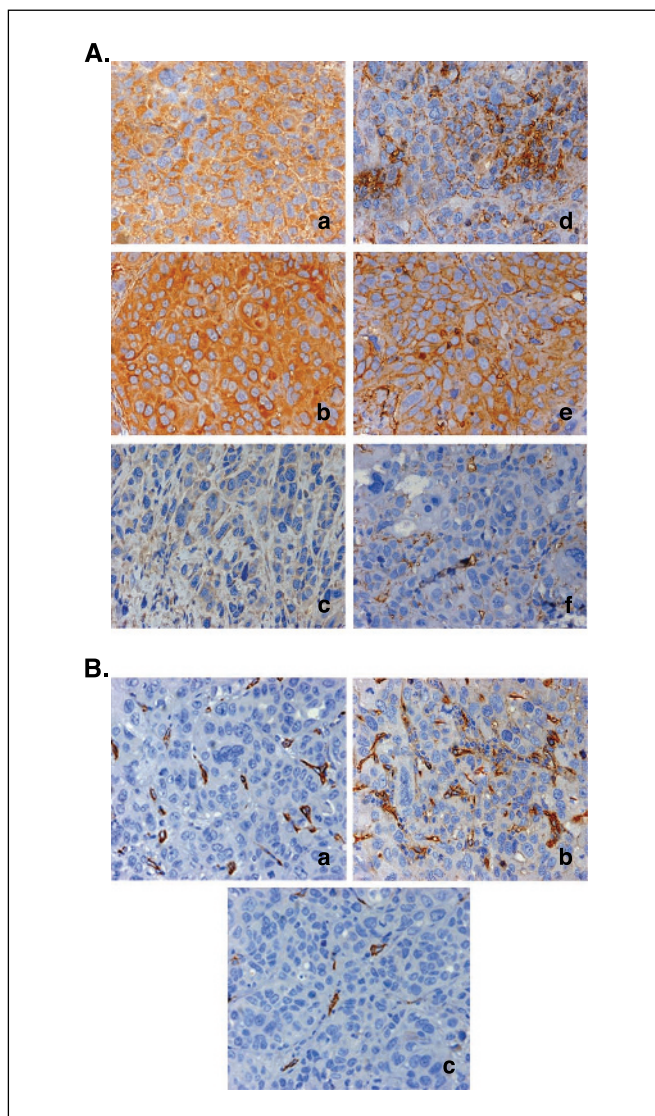


Figure 8. Localization of HYAL1, hyaluronic acid, and microvessels in tumor tissues. A, localization of HYAL1 and hyaluronic acid. a-c, HYAL1 localization; d-f, hyaluronic acid localization; a and d, vector transfectant; b and e, HYAL1-S transfectant; c and f, HYAL1-AS transfectant. B, localization of microvessels. Areas of the highest MVD from each type of tumor specimen. Magnification, $\times 400$. a, vector tumor; b, HYAL1-S tumor; c, HYAL1-AS tumor.

hyaluronic acid causes avascularity (49). Angiogenic hyaluronic acid fragments are present in the urine of grade 2 and 3 bladder cancer patients, suggesting that the hyaluronic acid and HYAL1 system is active in bladder cancer (19). Our observations that HYAL1-S tumors have a significantly higher MVD than vector tumors and HYAL1-AS tumors have the lowest MVD among the three tumor groups are consistent with the function of the tumor associated hyaluronic acid-hyaluronidase system. Jacobson et al. also observed increased MVD in HYAL1-overexpressing rat colon carcinoma xenografts (38).

At present, the role of hyaluronidase as a tumor promoter or a repressor has been controversial. The results presented in this study show that blocking HYAL1 expression reduces tumor growth and invasion. HYAL1 levels in various cancers are associated with high-grade invasive tumors (7, 8, 26, 27, 34). However, chromosome region 3p21.3 that contains *HYAL1*, *HYAL2*, and *HYAL3* genes is deleted in some cancer lines (50–53). Although the tumor suppressor gene in 3p21.3 is not *HYAL1*, *HYAL2*, or *HYAL3*, it originally gave rise to the idea that hyaluronidase is a tumor suppressor (54). Jacobson et al. found that the overexpression of HYAL1 by cDNA transfection in a rat colon carcinoma line decreases tumor growth, although the tumors are angiogenic (38). It is noteworthy that HYAL1-overexpressing transfectants in that study secreted 4- to 5-fold more hyaluronidase activity than the HYAL1-S transfectants in our study. Shuster et al. injected a large dose (300 or 75 units/injection \times 4 injections) of bovine testicular hyaluronidase in MDA435 breast cancer xenografts and observed a reduction in tumor volume over a period of 4 days; however, the effect was not studied beyond 4 days (55). Thus, it is possible that the effect of HYAL1 (and possibly other hyaluronidases) on tumor growth and invasion is concentration dependent. Although moderate HYAL1 expression in tumor cells increases their proliferative and invasive potentials, the lack of HYAL1 expression as well as very high HYAL1 expression decreases tumor growth and invasion perhaps

by completely degrading the tumor-associated hyaluronic acid matrix. It is also noteworthy that other proteins related to hyaluronic acid synthesis (hyaluronic acid synthase 2 and 3) and hyaluronic acid-receptor RHAMM are also involved in tumor growth and metastasis. For example, blocking hyaluronic acid synthase 3 expression in prostate cancer cells decreases cell growth *in vitro* and tumor growth *in vivo*. Hyaluronic acid synthase 2 expression induces mesenchymal and transformed properties in normal epithelial cells (56–58). Interestingly, hyaluronic acid synthase 2 expression in the absence of hyaluronidase decreases tumor growth in glioma cells. Interaction between RHAMM and hyaluronic acid fragments is known to induce the mitogen-activated protein kinase pathway, and overexpression of RHAMM is a useful prognostic indicator for breast cancer. These results show that the hyaluronic acid-hyaluronidase system is involved in the regulation of tumor growth and invasion.

Taken together, our study shows that HYAL1 is one of the molecular determinants of bladder tumor growth and invasion; therefore, it is a sensitive and specific marker for detecting high-grade bladder cancer.

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