HYAL1-v1, An Alternatively Spliced Variant of HYAL1 Hyaluronidase: A Negative Regulator of Bladder Cancer

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Abstract

Tumor cells express HYAL1 hyaluronidase, which degrades hyaluronic acid. HYAL1 expression in bladder cancer cells promotes tumor growth, invasion, and angiogenesis. We previously described five alternatively spliced variants of HYAL1 that encode enzymatically inactive proteins. The HYAL1-v1 variant lacks a 30-amino acid sequence that is present in HYAL1. In this study, we examined whether HYAL1-v1 expression affects bladder cancer growth and invasion by stably transfecting HT1376 bladder cancer cells with a HYAL1-v1 cDNA construct. Although HYAL1-v1 transfectants expressed equivalent levels of enzymatically active HYAL1 protein when compared with vector transfectants, their conditioned medium had 4-fold less hyaluronidase activity due to a noncovalent complex formed between HYAL1 and HYAL1-v1 proteins. HYAL1-v1 transfectants grew 3- to 4-fold slower due to cell cycle arrest in the G2-M phase and increased apoptosis. In HYAL1-v1 transfectants, cyclin B1, cdc2/p34, and cdc25c levels were >2-fold lower than those in vector transfectants. The increased apoptosis in HYAL1-v1 transfectants was due to the extrinsic pathway involving Fas and Fas-associated death domain up-regulation, caspase-8 activation, and BID cleavage, leading to caspase-9 and caspase-3 activation and poly(ADP-ribose) polymerase cleavage. When implanted in athymic mice, HYAL1-v1-expressing tumors grew 3- to 4-fold slower and tumor weights at day 35 were 3- to 6-fold less than the vector tumors ($P < 0.001$). Whereas vector tumors were infiltrating and had high mitoses and microvessel density, HYAL1-v1 tumors were necrotic, infiltrated with neutrophils, and showed low mitoses and microvessel density. Therefore, HYAL1-v1 expression may negatively regulate bladder tumor growth, infiltration, and angiogenesis. (Cancer Res 2006; 66(23): 11219-27)

Introduction

Hyaluronidase is a class of endoglycosidases that degrade hyaluronic acid. Hyaluronic acid is a glycosaminoglycan made up of repeating disaccharide units D-glucuronic acid and N-acetyl-D-glucosamine. Hyaluronic acid, in addition to its structural role, regulates cell adhesion, migration, and proliferation by interacting with the cell surface receptors (CD44 and RHAMM; refs. 1–3). Hyaluronic acid concentration is elevated in several tumors and with the cell surface receptors (CD44 and RHAMM; refs. 1–3).

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We have shown that tumor cells express HYAL1-type hyaluronidase (16). The increased hyaluronidase levels in bladder cancer patients are due to increased HYAL1 expression in bladder tumor cells both at the transcriptional and protein levels (16–18). HYAL1 expression is a potential prognostic indicator for prostate cancer progression and a possible marker for detecting head and neck squamous cell carcinoma (19–21). We have recently shown that HYAL1 is one of the molecular determinants of tumor growth, infiltration, and angiogenesis (22, 23). For example, blocking HYAL1 expression in bladder and prostate cancer cells by antisense cDNA transfection decreased cell proliferation, caused cell cycle arrest (G2-M phase), and reduced the invasive activity of tumor cells (22, 23). In xenografts, HYAL1-antisense transfectant tumors grew 9- to 17-fold slower, resembled benign neoplasia (i.e., no infiltration into skeletal muscle, lymph node, and blood vessels), and had reduced microvessel density when compared with the vector tumors (22, 23). Interestingly, HYAL1 expression in tumor cells by cDNA transfection at levels (i.e., hyaluronidase levels >100 milliunits/mL) much higher than those normally expressed in tumor cells, tumor tissues, and in urine induces apoptosis and prevents tumor formation (23).

In the human genome, there are six hyaluronidase genes that occur in two clusters: on chromosomes 3p21.3 (HYAL1, HYAL2, and HYAL3) and 7q31.3 (PH20, HYALP1, and HYAL4; ref. 24). Among these six hyaluronidases, HYAL1, HYAL2, and PH20 are well characterized. PH20, or the testicular hyaluronidase, is expressed in laryngeal carcinoma (25). At the present time, very little is known about the regulation of hyaluronidase expression in normal and tumor tissues. However, using normal and tumor tissues and cells, we cloned five splice variants of HYAL1 generated by alternative mRNA splicing. These splice variants encode truncated HYAL1 proteins that are enzymatically inactive (26). We also cloned similar splice variants of HYAL3 (26). The functions of the splice variants of either HYAL1 or HYAL3 are unknown. Among various splice variants of HYAL1, HYAL1-v1 encodes a protein that lacks only a 30-amino acid sequence (amino acids 301-330), which is present in the wild-type HYAL1 protein that contains 435 amino acids. This 30-amino acid sequence, which is encoded by a single exon (i.e., exon 2) in the HYAL1 transcript, is well conserved in all six human hyaluronidases and in the bee hyaluronidase (26).

In this study, we transfected bladder cancer cells with a full-length HYAL1-v1 cDNA construct and examined the phenotype of carcinomas (4–10). For example, we and others have shown that urinary hyaluronic acid levels serve as an accurate diagnostic marker for detecting bladder cancer and for monitoring its recurrence (6, 9–12). Hyaluronidase degrades hyaluronic acid into small fragments, some of which are angiogenic (13, 14). Hyaluronidase levels are elevated in the urine of patients with high-grade bladder cancer, and the hyaluronidase test detects these patients with ~85% accuracy (6, 10, 11, 15).
these transfectants both in *vitro* and in xenografts. Our results show that HYAL1-v1 expression leads to decreased proliferation of bladder cancer cells, and this is caused by cell cycle arrest and induction of apoptosis. In xenografts, HYAL1-v1-expressing tumors grow slower and are less angiogenic and invasive.

**Materials and Methods**

**Transfection.** HYAL1-v1 cDNA containing the entire coding region (Genbank accession no. AF502904) was PCR amplified using a HYAL1-v1 cDNA construct (26) and the following PCR primers: forward (110-129; AF502904), 5'TTGTTCCTCGAGCAGTTCGGC3; reverse (1,351-1,332; AF502904), 5'ATCCACATGCTCTTCCG3'. In the reverse primer, we introduced an "A" to "C" change in the fourth position so that, in the PCR-amplified cDNA, the stop codon TGA will be replaced by GGA that codes for glycine. The 1.3-kb PCR-amplified product was cloned into the pEF6/v5-His eukaryotic expression vector (Invitrogen, Carlsbad, CA). Due to the elimination of the stop codon, the HYAL1-v1 protein will fuse with the v5 epitope (a 9-amino acid sequence), encoded by the vector sequence.

**Analysis of hyaluronidase activity and HYAL1 and HYAL1-v1 expression.** Hyaluronidase activity secreted in the serum-free conditioned medium (RPMI 1640 plus insulin, transferrin, and selenium supplement plus gentamicin) of transfectants was analyzed by the hyaluronidase ELISA-like assay (27), and the hyaluronidase activity (milliunits/mL) was normalized to cell number. Conditioned medium from 5 x 10⁴ cells were concentrated and separated on a substrate (hyaluronic acid gel) to detect active hyaluronidase species (27). Serum-free conditioned media (5 x 10⁴ cells) were subjected to HYAL1 immunoblot analysis using a rabbit anti-HYAL1 IgG (16). For HYAL1-v1 immunoblotting, either a rabbit anti-HYAL1-v1 IgG (26) or an anti-v5 monoclonal antibody (Invitrogen) was used. Alternatively, HYAL1 was immunoprecipitated from the serum-free conditioned medium using the anti-HYAL1 IgG and the immunoprecipitates were immunoblotted using the anti-v5 IgG. As a loading control, the blots were reprobed with a horseradish peroxidase–conjugated goat anti-human transferrin antibody (Bethyl Laboratories, Montgomery, TX).

**In vitro translation.** HYAL1 and HYAL1-v1 cDNAs were in *vitro* translated using the TNT Quick Coupled Transcription/Translation System (Promega, Madison, WI) and 20 µCi of Redivue 1-35S methionine (GE Healthcare, Piscataway, NJ) or unlabeled methionine (26). Aliquots of the [35S]methionine-labeled samples were analyzed by SDS-PAGE and fluorography. Translation mixture containing [35S]methionine-labeled HYAL1 was incubated with HYAL1 IgG-conjugated Sepharose beads or normal rabbit IgG-conjugated beads, and the immunoprecipitates were analyzed by SDS-PAGE and fluorography. HYAL1 IgG and normal rabbit IgG beads were prepared by coupling the respective antibodies to CNBr-activated Sepharose beads (GE Healthcare). To analyze the HYAL1 and HYAL1-v1 interaction, the translation mixture containing unlabeled HYAL1 was immunoprecipitated using the HYAL1 IgG beads or normal rabbit IgG beads. The beads were washed in radioimmunoprecipitation assay (RIPA) buffer (50 mmol/L Tris- HCl, 150 mmol/L NaCl, pH 7.4), 0.2% Triton X-100, 0.1% bovine serum albumin, protease inhibitors and then incubated with the translation mixture containing [35S]methionine-labeled HYAL1-v1. Following incubation at 4°C for 16 hours, the beads were washed in RIPA buffer and the protein bound to beads was analyzed by SDS-PAGE and fluorography.

**Cell proliferation, cell cycle, and apoptosis assays.** For the cell proliferation assay, transfectants plated on 24-well plates in growth medium plus basicidin were counted every 24 hours for a total of 120 hours. Cell cycle phase distribution in actively growing transfected cultures was estimated by propidium iodide staining of DNA followed by flow cytometry (22, 23). For the apoptosis assay, 96-hour cultures of transfectants were analyzed using the Cell Death ELISA Plus kit (Roche Diagnostics, Pleasanton, CA). Mitochondrial depolarization was examined by incubating actively growing cultures of transfectants with a mitochondria-specific dye, JC-1, for 15 minutes followed by flow cytometry (23, 28).

To study the effect of angiogenic hyaluronic acid fragments on cell proliferation and apoptosis, transfectants were cultured in growth medium plus basicidin and various concentrations (0-50 µg/mL) of a mixture of hyaluronic acid fragments (15, 8, and 2 kDa; Genzyme Corp., Cambridge, MA). Following 96 hours of incubation, the cells were counted or subjected to apoptosis assay.

**Matrigel invasion assay.** Transfectants (3 x 10⁴ cells) were plated in the upper chamber of a Matrigel-coated Transwell (12-µm pore) plate in serum-free medium. The bottom chamber contained growth medium. After 48 hours, invasion of cells in the bottom chamber was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cell invasion was quantified as a ratio of the number of cells in the bottom chamber divided by the total number of cells in the top and bottom chamber and then expressed as a percentage (22, 23).

**Immunoblot analysis.** Cell lysates (4 x 10⁴ cells) were immunoblotted using anti-cyclin B1, anti-cdc2/p34, anti-cdc25c, anti-chk1, anti-wee1, anti-active caspase-3, anti-cleaved poly(ADP-ribose) polymerase (PARP; Asp⁴⁺), anti-caspase-9, anti-Fas, anti-Fas ligand (Fas-L), anti-Fas-associated death domain (FADD), anti-caspase-8, and anti-BID antibodies (22, 29). Various antibodies were purchased from NeoMarkers (Fremont, CA; cell cycle–related IgGs), Santa Cruz Biotechnology, Inc. (Santa Cruz, CA; Fas-L and cdc25c IgGs), Cell Signaling (Danvers, MA; cleaved PARP), and BD Biosciences (San Jose, CA; anti-caspase-3, caspase-9, caspase-8, Fas, Fadd, and BID IgGs).

**Fas small interfering RNA transfection.** The ON-TARGETplus SMARTpool small interfering RNA (siRNA) against Fas and ON-TARGETplus siCONTROL nontargeting siRNA were obtained from Dharmacon Research (Boulder, CO). HT1376 cells (4 x 10⁴ per 24-well plates) were transiently transfected with Fas or control siRNA (50 nmol/L) using LipofectAMINE RNAiMAX reagent as per the manufacturer’s instructions (Invitrogen; ref. 30). Twenty-seven hours after transfection, the cells were either counted or subjected to apoptosis assay or to anti-Fas IgG immunoblotting.

**Tumor xenografts.** Transfectants (2 x 10⁴ cells) were s.c. implanted on the dorsal flank of 5-week-old mice (five animals per clone). Time for the tumors to become palpable was noted. Tumor size was measured twice weekly, and tumor volume was calculated by approximating the tumor to an ellipsoid (22, 23, 28). At necropsy, tumors were weighed and Tukey’s multiple comparison test was used to compare the differences in tumor growth rate and tumor weight. Tumor histology was done at Charles River Laboratories.

**Localization of HYAL1-v1 and microvessel density determination.** HYAL1-v1 and microvessels were localized in 3-µm sections of tumor specimens by immunohistochemistry (6, 19, 22). HYAL1-v1 IgG (1:1,000 dilution) and a rat anti-mouse CD34 IgG (3.1 µg/mL) were used for HYAL1-v1 and CD34 localization (i.e., to visualize microvessels), respectively. Microvessel density and the length of the microvessels were determined as described previously (22, 23).

**HYAL1-v1 reverse transcription-PCR.** Total RNA was extracted from six normal bladder and six bladder tumor tissues (low grade, n = 2; high grade, n = 4). The RNA was subjected to reverse transcription-PCR (RT-PCR) to amplify the HYAL1-v1 transcript (26) using the following primers: forward (110-129; AF502904), 5’TTGTTCCTCGAGCAGTTCGGC3; reverse (1,351-1,332; AF502904), 5’ATCCACATGCTCTTCCG3’. PCR products were separated on a 1.2% agarose gel and the intensity of the HYAL1-v1 PCR bands was determined using the Kodak 1D Gel Analysis software. The intensity of the bands was normalized to β-actin PCR product, which was amplified by RT-PCR (32 PCR cycles) using the following primers: forward (75-94; BC014861) and reverse (835-816; BC014861) 5’GCGTCTATGG-CATTGGTGT-3’. The mean HYAL1-v1/β-actin ratio between normal and bladder tumor tissues was compared using the unequal t test.

**Results**

**Analyses of HYAL1 and HYAL1-v1 expression in HT1376 transfectants.** HT1376 cells were stably transfected with vector.
or the HYAL1-v1 construct. Twenty-five to 30 clones of each vector and HYAL1-v1 transfectants were analyzed for hyaluronidase activity and HYAL1 and HYAL1-v1 expression analyses. Data on two vector and five HYAL1-v1 clones are presented. We have previously shown that HYAL1-v1 protein is enzymatically inactive (26), and therefore, we expected no change in hyaluronidase activity secreted by vector and HYAL1-v1 transfectants. However, as shown in Fig. 1A, vector clones secrete ~4-fold higher hyaluronidase activity than HYAL1-v1 clones, but immunoblotting with anti-HYAL1 IgG shows no change in HYAL1 protein expression in vector and HYAL1-v1 transfectants (Fig. 1B, a).

Immunoblot analysis using the anti-HYAL1-v1 IgG shows that HYAL1-v1 protein is expressed in HYAL1-v1 transfectants but not in vector transfectants (Fig. 1B, b). The HYAL1-v1 protein expressed in HYAL1-v1 transfectants is a HYAL1-v1/v5 fusion protein (i.e., HYAL1-v1 protein fused to the 9-amino acid v5 epitope) because it

Figure 1. Analysis of hyaluronidase activity and HYAL1 and HYAL1-v1 expression in HT1376 transfectants. A, measurement of hyaluronidase (HAase) activity (milliunits/10^5 cells) by hyaluronidase ELISA-like assay. Columns, mean hyaluronidase activity from three separate experiments (triplicate measurement/experiment); bars, SE. B, a to c, immunoblot analysis. Conditioned media from 5 × 10^5 cells of each transfectant clone were subjected to anti-HYAL1 (a), anti-HYAL1-v1 (b), and anti-v5 (c) immunoblotting. d, substrate [hyaluronic acid (HA)] gel assay. Conditioned media (5 × 10^5 cells) from transfectants were subjected to substrate (hyaluronic acid) gel assay. In the absence of a well-accepted protein loading control for secreted proteins, we used the cell number to normalize the amount of conditioned medium used in the hyaluronidase ELISA-like, immunoblot, and substrate (hyaluronic acid) gel assays. e, the blots or conditioned media were subjected to anti-transferrin immunoblotting. f, conditioned media of transfectants (2 × 10^5 cells) were immunoprecipitated using the anti-HYAL1 antibody and then immunoblotted using the anti-v5 antibody. As controls, equivalent amounts of conditioned media of HYAL1-v1 clones 4 and 5 were immunoprecipitated using normal rabbit IgG and then blotted using the anti-v5 antibody. As a loading control, before subjecting it to anti-HYAL1-v1 immunoblotting, the blot was stained with Coomassie blue and then destained to visualize antibody bands. C, analysis of in vitro–translated HYAL1 and HYAL1-v1. HYAL1 and HYAL1-v1 cDNAs were in vitro transcribed and translated in the presence of [35S]methionine and then subjected to SDS-PAGE and fluorography. Lane 1, HYAL1; lane 2, HYAL1-v1. [35S]methionine-labeled HYAL1-v1 product was bound to HYAL1 IgG beads that were first incubated with HYAL1 (lanes 3 and 4) or HYAL1 IgG beads alone (lane 5) or normal rabbit IgG beads (lane 6). [35S]methionine-labeled HYAL1 was immunocaptured using either HYAL1 IgG (lane 7) or normal rabbit IgG (lane 8) beads.
is detected by anti-v5 IgG immunoblotting (Fig. 1B, c). Interestingly, the hyaluronidase ELISA-like assay showed that HYAL1-v1 transfectants secrete ~4-fold less hyaluronidase activity than vector transfectants; no decrease in hyaluronidase activity was observed in the substrate (hyaluronic acid) gel assay (Fig. 1B, d). The anti-transferrin immunoblotting shows that the differences in HYAL1-v1 expression and hyaluronidase activity among vector and HYAL1-v1 transfectants are not due to the differences in sample loading (Fig. 1B, e).

Immunoprecipitation of HYAL1 protein from the conditioned medium by anti-HYAL1 IgG followed by immunoblotting using anti-v5 IgG shows that HYAL1-v1 protein is present in the conditioned medium of HYAL1-v1 transfectants (Fig. 1B, f). This suggests that the noncovalent complex formation between HYAL1-v1 with HYAL1 possibly decreases the hyaluronidase activity of HYAL1 protein. Decreased hyaluronidase activity in HYAL1-v1 transfectant conditioned medium was not due to the presence of v5 epitope on the HYAL1-v1 protein because stable transfectants expressing HYAL1-v5 fusion protein overexpressed hyaluronidase activity (25-40 milliunits/10^5 cells) when compared with the vector transfectants (data not shown).

To examine whether there is a direct interaction between HYAL1 and HYAL1-v1, we evaluated the interaction between recombinant HYAL1 and HYAL1-v1 proteins generated by in vitro translation using a HYAL1 IgG pull-down assay. As shown in Fig. 1C, the in vitro–translated HYAL1 and HYAL1-v1 polypeptides are of the expected molecular mass, approximately 50 and 47 kDa, respectively (26). Figure 1C also shows that the HYAL1-v1 polypeptide binding HYAL1 IgG immunoaffinity beads, which were first incubated with HYAL1 polypeptide. However, neither the HYAL1 IgG beads (not preincubated with the HYAL1 polypeptide) nor the normal rabbit IgG beads captured the HYAL1-v1 polypeptide. These data suggest a direct interaction between HYAL1 and HYAL1-v1. The specificity of HYAL1 IgG beads to immunocapture the HYAL1 polypeptide is also shown in Fig. 1C.

Effect of HYAL1-v1 expression on cell proliferation and cell cycle. As shown in Fig. 2A, all five HYAL1-v1 transfectants grew 3- to 4-fold slower than vector transfectants. The doubling time of vector transfectants was ~26 hours, whereas that of HYAL1-v1 transfectants was 72 to 96 hours. To determine whether the observed decrease in cell proliferation among HYAL1-v1 transfectants was a result of the reduced hyaluronidase activity, we exposed the vector and HYAL1-v1 transfectants to a mixture of angiogenic hyaluronic acid fragments. As shown in Fig. 2B, the hyaluronic acid fragments caused a dose-dependent but modest increase (125-190%) in cell proliferation. This result shows that angiogenic hyaluronic acid fragments can partially rescue the growth inhibition observed in HYAL1-v1 transfectants.

As shown in Table 1, HYAL1-v1 expression seems to cause a G2-M arrest in the cell cycle. There is 132% to 172% increase in the number of HYAL1-v1 transfectants in the G2-M phase when compared with the vector transfectants (P = 0.023, t test). Correspondingly, there is a decrease in the percentage of HYAL1-v1 cells in the S phase when compared with the vector transfectants.
Next, we examined the expression of G2-M regulators (i.e., cdc2/c, cdc2/p34, cyclin B1, chk1, and wee1) in various transfectant clones by immunoblotting. As shown in Fig. 2C, there is >2-fold decrease in cyclin B1, cdc2/p34, and cdc2/c expression in HYAL1-v1 transfectants when compared with the vector transfectants. However, no change in the expression of negative regulators of the G2-M phase (i.e., wee1 and chk1) was observed among vector and HYAL1-v1 transfectants (Fig. 2C). These results show that HYAL1-v1 transfectants are arrested in the cell cycle due to a down-regulation of some of the positive regulators of the G2-M phase.

**Effect of HYAL1-v1 expression on apoptosis.** We examined whether the slower growth of HYAL1-v1 transfectants is also due to induction of apoptosis. As shown in Fig. 3A, there is a 3- to 5-fold increase in the apoptotic activity in HYAL1-v1 clones when compared with vector clones ($P < 0.001$, Tukey’s multiple comparison test). As shown in Fig. 3B, the exposure of HYAL1-v1 transfectants to an angiogenic hyaluronic acid fragment mixture caused only a modest decrease (24-45%) in apoptosis. This suggests that the angiogenic hyaluronic acid fragments rescue the HYAL1-v1 phenotype only modestly.

To further confirm that HYAL1-v1 transfectants are more apoptotic, we examined caspase-3 and caspase-9 activation. As shown in Fig. 3C, activated caspase-3 is detected in HYAL1-v1 transfectants and in staurosporine-treated HT1376 cells (positive control) but not in vector transfectants. Similarly, cleaved caspase-9 was detected in both HYAL1-v1 transfectants and

![Figure 3](image_url)

**Figure 3.** Examination of apoptosis. A, apoptotic activity in various transfectant clones was measured using the Cell Death ELISA Plus assay. Columns, mean (triplicates in two experiments); bars, SD. B, effect of angiogenic hyaluronic acid fragments on apoptosis in HYAL1-v1 transfectants. Points, mean; bars, SD. C, immunoblot analysis of apoptotic pathway proteins. Cell lysates of HT1376 transfectants were analyzed by immunoblot analysis using IgGs against active caspase-3 (a), caspase-9 (b), anti-cleaved PARP (c), caspase-8 (d), Fas (e), Fas-L (f), FADD (g), BID (h), and actin (i). Positive controls: staurosporine-treated (1 μmol/L/4 hours) HT1376 cells (a and b); Jurkat cell lysates (c and e); HL-60 cell lysates (f); A431 cell lysates (g). Jurkat, HL-60, and A431 cell lysates were obtained from Santa Cruz Biotechnology. D, immunoblot analysis of Fas. Cell lysates of HYAL1-v1 transfectants (1-4) transiently transfected with Fas or control siRNA were subjected to immunoblotting using anti-Fas IgG. E, apoptotic activity in HYAL1-v1 transfectants transiently transfected with Fas or control siRNA. Columns, mean; bars, SD.
Caspase-8 activation, Fas, Fas-L, caspase-3 activation, leading to the abnormal cleavage of PARP effectants is induced by the receptor-mediated (Fas/Fas-L) or the apoptosis. In HYAL1-v1 transfectants, although they are undergoing significant increase in mitochondrial depolarization was observed in HYAL1-v1 transfectants. This indicates that HYAL1-v1 transfectants have intact mitochondria, although they are undergoing apoptosis.

We investigated whether the apoptosis in HYAL1-v1 transfectants is induced by the receptor-mediated (Fas/Fas-L) or the extrinsic pathway. This pathway involves the formation of the death-inducing signaling complex (DISC) and FADD-mediated activation of caspase-8, which in turn causes BID cleavage and caspase-3 activation, leading to the abnormal cleavage of PARP (31, 32). We therefore examined caspase-8 activation, Fas, Fas-L, and FADD levels and BID cleavage. As shown in Fig. 3C, d, caspase-8 activation (i.e., cleaved caspase-8) and 2- to 3-fold up-regulation of Fas and FADD (Fig. 3C, e and g) are observed in all five HYAL1-v1 transfectant clones when compared with vector clones. However, no change in Fas-L expression was observed among vector and HYAL1-v1 transfectants (Fig. 3C, f). As expected from the caspase-8 activation, BID cleavage was observed in four of five HYAL1-v1 transfectant clones based on the appearance of a 15-kDa cleaved BID band (Fig. 3C, h).

To establish that HYAL1-v1 transfectants are undergoing apoptosis via the receptor-mediated (Fas/Fas-L) or the extrinsic pathway, we examined whether blocking Fas expression by transiently transfecting HYAL1-v1 transfectants with Fas siRNA (30) will reduce apoptosis. As shown in Fig. 3D, the transfection of Fas siRNA decreased Fas expression in HYAL1-v1 transfectants. Furthermore, following Fas siRNA transfection, there was a 2- to 3-fold decrease in apoptosis in all four HYAL1-v1 transfectant clones (Fig. 3E). These results show that HYAL1-v1 expression in HT1376 cells most likely induces apoptosis via the extrinsic pathway.

Effect of HYAL1-v1 expression on invasive activity. Invasive activity of the vector and HYAL1-v1 transfectants was examined by Matrigel invasion assay. HYAL1-v1 clones 1 (7.3 ± 3.5%) and 3 (11.6 ± 2.7%) were less invasive than vector clones (1, 27.8 ± 1.5%; 2, 33.5 ± 0.6%). However, the invasive activity of HYAL1-v1 clones 2 (33.4 ± 5.4%), 4 (26.3 ± 3.2%), and 5 (26.4 ± 4.5%) was not very different from that of the vector transfectants. Therefore, HYAL1-v1 expression does not significantly affect the invasive phenotype of HT1376 cells when tested in vitro.

Effect of HYAL1-v1 expression on tumor xenografts. As shown in Fig. 4A, there was a 3- to 4-fold delay in the generation of palpable s.c. tumors in the animals injected with HYAL1-v1 transfectants when compared with the animals injected with the vector clones (palpable tumors, 7-10 days; P < 0.001). The 3- to 6-fold decrease in the weight of HYAL1-v1 tumors when compared with vector tumors is also statistically significant (P < 0.001, Tukey’s multiple comparison test; Fig. 4B). These results show that HYAL1-v1 expression in HT1376 bladder cancer cells decreases tumor growth.

The histology report and the photomicrographs showed high mitoses in vector tumors (8-10 mitotic figures/high-power field) when compared with HYAL1-v1 tumors (0-3 mitotic figures/high-power field; Fig. 4C). As shown in the photomicrographs, HYAL1-v1 tumors are necrotic and contain tumor cells with small dark pyknotic nuclei (representing additional necrosis). For example, whereas 0% to 15% of the cross-sectional areas in vector tumors were necrotic, necrosis was evident in ≥75% of the cross-sectional areas in HYAL1-v1 tumors. In addition, there was moderate (Fig. 4C, c) to heavy (Fig. 4C, f) neutrophil infiltration in HYAL1-v1 tumors, whereas there was minimal (if any) neutrophil infiltration in vector tumors. In this study, we did not examine the metastatic ability of HYAL1-v1 tumors because HYAL1-v1 expression did not consistently affect invasion in Matrigel assays. However, the histology of the primary tumors showed no invasion of tumor cells into the skeletal muscle in HYAL1-v1 tumor specimens (Fig. 4C), but vector tumors infiltrated skeletal muscle. This suggests that HYAL1-v1 tumors seem to be minimally invasive.

Immunohistochemical analysis shows high level expression of HYAL1-v1 in HYAL1-v1 tumor specimens, and it is expressed exclusively in tumor cells (Fig. 5A). Very little HYAL1-v1 expression is observed in vector tumors. Microvessel density is higher in vector tumor specimens when compared with HYAL1-v1 specimens. Furthermore, the blood vessels observed in vector specimens are much longer than those in HYAL1-v1 specimens (Fig. 5B). Quantification of microvessels in various specimens shows that the microvessel density in vector specimens is 2- to 7-fold higher than that in HYAL1-v1 specimens (Fig. 5C). Similarly, the microvessels in vector tumors are 3- to 5-fold longer than the microvessels found in HYAL1-v1 tumors (Fig. 5D).

Expression of HYAL1-v1 transcript in bladder tissues. We examined whether there is a differential expression of the HYAL1-v1 transcript in normal and bladder tumor tissues using RT-PCR analysis. As shown in Fig. 6, there is higher expression of HYAL1-v1 transcript in normal bladder tissues when compared with bladder tumor tissues. The mean HYAL1-v1/β-actin ratio in normal bladder tissues (0.84 ± 0.14) was 2.3-fold higher than that in bladder tumor tissues (0.37 ± 0.21). The difference in the HYAL1-v1/β-actin ratio between normal and bladder tumor tissues was statistically significant (P = 0.0062, unpaired t test). These data support the role of HYAL1-v1 as a negative regulator of bladder tumor growth and progression.

Discussion
The results of our study show that the expression of a HYAL1 variant protein, HYAL1-v1, in bladder cancer cells decreases cell growth, causes G2-M arrest, and induces apoptosis. In xenograft studies, HYAL1-v1 expression decreases tumor growth and inhibits angiogenesis and infiltration into local skeletal muscle fibers. HYAL1-v1 is generated by alternative splicing of HYAL1 mRNA, in which a 90-bp region (nucleotides 1,520-1,610; Genbank accession no. AF50294) is deleted. We have previously reported that these 90 bases make up the entire exon 2, and therefore, HYAL1-v1 is generated by splicing of exon 2 (26). It is noteworthy that the 30-amino acid sequence encoded by exon 2 is not a part of the catalytic domain; however, it is well conserved in all six human hyaluronidases and also in the bee hyaluronidase (33–36). The observation that HYAL1-v1 is enzymatically inactive and that a
similar splice variant of HYAL-3 (HYAL3-v1) is also enzymatically inactive may explain why this 30-amino acid sequence that is encoded by a separate exon in both HYAL1 and HYAL3 is well conserved (26).

Our data on the RT-PCR analysis of the HYAL1-v1 transcript in bladder tissues show that HYAL1-v1 expression is lower in bladder tumor tissues when compared with normal bladder tissues. These data are consistent with the negative regulatory effect of HYAL1-v1 expression on cell growth, tumor invasion, and angiogenesis.

We are currently examining HYAL1-v1 expression using tissue microarrays and immunohistochemistry to confirm the RT-PCR data.

We recently showed that the production of enzymatically active HYAL1 by bladder cancer cells is necessary for cell growth, cell cycle progression, and invasive activity in vitro and tumor growth, infiltration, and angiogenesis in vivo. If all of the effects of HYAL1 on tumor cells are due to its effect on hyaluronic acid degradation, then expression of HYAL1-v1 in HT1376 cells should have
been functionally silent. This is because HYAL1-v1 is enzymatically inactive, and in this study, all of the HYAL1-v1 transfectants expressed HYAL1 at levels similar to those expressed by the vector transfectants. However, at least some of the effects of HYAL1-v1 are mediated through the attenuation of HYAL1 activity because HYAL1-v1/HYAL1 complex is most likely enzymatically inactive because, by the hyaluronidase ELISA-like assay, HYAL1-v1 transfectants are found to secrete 3- to 4-fold less hyaluronidase activity when compared with the vector transfectants. In this regard, HYAL1-v1 transfectants behave like HYAL1-antisense transfectants, which are also blocked in the G2-M phase of the cell cycle (22). In addition, like HYAL1-antisense transfectants, HYAL1-v1 transfectants form tumors that are less angiogenic (i.e., decreased microvessel density and shorter lengths of tumor microvessels). Therefore, it is possible that, in HYAL1-v1 tumors, decreased hyaluronidase activity causes decreased production of angiogenic hyaluronic acid fragments, which in turn resulted in decreased angiogenesis. However, because hyaluronidase limits tumor growth at the present time, it is unclear whether the lack of angiogenesis is limiting tumor growth or the lack of tumor growth is limiting angiogenesis.

At the present time, it is unclear how HYAL1-v1 expression in bladder cancer cells induces apoptosis via the death receptor pathway that involves DISC formation. It is noteworthy that we consistently observed a higher percentage of floating cells in the

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**Figure 5.** Tumor histology, HYAL1-v1 immunohistochemistry, and microvessel density determination in tumor specimens. A, HYAL1-v1 localization by immunohistochemistry. a and b, vector clones 1 and 2; c to f, HYAL1-v1 clones 1, 2, 3, and 4, respectively. Magnification, ×400. B, localization of microvessels. The areas of highest microvessel density are presented at ×400 magnification. a and b, vector clones 1 and 2; c to f, HYAL1-v1 clones 1, 2, 3, and 4, respectively. C, microvessel density (MVD) in vector and HYAL1-v1 transfectant tumors. Microvessels were counted in 10 random high-power fields representing the highest microvessel density per tumor specimen. Columns, mean microvessel density; bars, SD. D, length of microvessels in vector and HYAL1-v1 transfectant tumors. The lengths of >25 microvessels were measured in 10 different high-power fields in each specimen. Columns, mean length; bars, SD.

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**Figure 6.** RT-PCR analysis of HYAL1-v1 expression in bladder tissues. Normal bladder (1-6) and bladder tumor (1 and 2, low grade; 3-6, high grade) were subjected to semiquantitative RT-PCR using either HYAL1-v1 primers (PCR cycles = 40; A) or actin primers (PCR cycles = 32; B). bp, pGEM DNA markers (Promega).
HYAL1-v1 Functions in Bladder Cancer


HYAL1-v1, An Alternatively Spliced Variant of HYAL1 Hyaluronidase: A Negative Regulator of Bladder Cancer

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