Expression of EphA2 and Ephrin A-1 in Carcinoma of the Urinary Bladder

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Abstract

Purpose: The EphA2 receptor tyrosine kinase is believed to play a role in tumor growth and metastasis. The clinical significance of the expression of EphA2 was observed in breast, prostate, colon, skin, cervical, ovarian, and lung cancers. The purpose of this work was to determine the expression of EphA2 and its ligand, Ephrin A-1, and E-cadherin in carcinoma of the urinary bladder, and determine EphA2 as a new target for therapy in bladder cancer.

Experimental Design: EphA2 mRNA and protein expression was investigated by reverse transcription-PCR and Western blot, respectively, in bladder cancer cell lines. In addition, the expression of EphA2, Ephrin A-1, and E-cadherin in tissues from patients with different stages of urinary bladder cancer was determined by immunohistochemistry. Furthermore, the ability of Ephrin A-1 to inhibit growth of bladder cancer cells was also investigated using an adenoviral delivery system.

Results: Western blot analysis showed high EphA2 expression in TCCSUP, T24, and UMUC-3 cell lines. In tissues, the staining intensity of EphA2 was less in normal urothelium but increased greatly in advancing stages of urothelial carcinoma ($P < 0.05$). Similarly, the staining intensity of Ephrin A-1 was low in normal tissues and high in cancerous tissues, but it was similar across the various stages of urothelial carcinoma (T1–T4). E-cadherin immunoreactivity decreased in urothelial cancer. Association of EphA2 and Ephrin A-1 expression was found to be significant between T1 stage and T2 stage ($P < 0.04$) and T2 and T3–T4 stages ($P < 0.0001$). Adenovirus delivery of Ephrin A-1 inhibited proliferation of TCCSUP cells.

Conclusion: EphA2 may serve as a novel target for bladder cancer therapy.

Urinary bladder cancer is diagnosed in at least 54,000 people each year in the United States and 14,000 people die annually from the disease (1, 2). Approximately 90% of the urinary bladder cancers are carcinomas that typically occur either as superficial (low-grade) or muscle-invasive (intermediate to high-grade) carcinoma. At the time of diagnosis, 75% of urothelial carcinomas are superficial papillary tumors and 25% are muscle-invasive cancers. Superficial tumors can be surgically resected; however, local recurrence is common and 20% to 30% of recurrent lesions progress to higher grade or stage. Muscle-invasive cancers have the greatest propensity to metastasize and are fatal in ~50% of the patients (3). Currently, the most reliable prognostic factors for recurrence and progression are tumor stage and grade (4, 5). The most common treatment strategies are transurethral resection combined with immunotherapy for superficial carcinoma (6) and cystectomy and chemotherapy/radiation therapy for invasive carcinoma (7). However, the limited success of the above prognostic and therapeutic strategies warrants the identification of new molecules that might provide much-needed targets for bladder cancer prognosis and treatment.

Receptor tyrosine kinases have been reported to regulate the growth and survival of several types of cancer and to provide signals necessary for tumor growth and metastasis (8). Recently, therapeutic targeting of receptor tyrosine kinases (such as epidermal growth factor receptor or Her2/neu) has been proved successful for clinical application to cancer (9). The Eph receptors encompass the largest family of receptor tyrosine kinases in the human genome and regulate many aspects of normal and diseased cell behaviors (10–12). The biological activity of Eph receptors is regulated by binding to ligands, known as ephrins, which are attached to the cell membrane. Structurally, the Eph receptors and ephrins have been classified into two subfamilies, A and B, based on their sequence conservation and binding (13). The Eph receptors bind preferentially to specific ephrins and cascade highly specific signaling at sites of interactions. Cross-talk is limited to within A and B subfamilies (14). Eph-ephrin binding has been linked to diverse functions, including intercellular interactions at...
segmental boundaries, neural path-finding, neural crest cell migration, remodeling of embryonic arteries and veins, signaling, and malignant growth and survival (15).

The EphA2 receptor tyrosine kinase was initially isolated as an epithelial cell kinase from a cervical carcinoma (HeLa) cDNA library (16) whereas Ephrin A-1 was identified as a factor induced by tumor necrosis factor α (17). In nontransformed cells, the binding of EphA2 to Ephrin A-1 promotes autophosphorylation of EphA2, which in turn triggers downstream signals that negatively regulate cell growth and migration, and also promotes rapid turnover of phosphorylated EphA2 (18, 19). Consequently, EphA2 is generally phosphorylated and found at low levels in nontransformed cells (20). In contrast, high levels of unphosphorylated EphA2 have been observed in melanoma (21) and cancers of the breast (20), colon (22), lung (23), prostate (24, 25), and esophagus (26). In many cancers, high levels of EphA2 predict metastasis and poor survival (20, 22–24). Consistent with a suggested role in malignant behavior, overexpression of EphA2 has been reported to induce malignant transformation of a nontransformed cell line (20). High levels of EphA2 are also observed in endothelial cells at sites of tumor neovascularization and targeted inhibition of EphA2-ligand binding in endothelial cells decreased angiogenesis and tumor progression (27). Expression of Ephrin A-1 is also observed in tumor endothelial cells and tumor cells and shown to promote endothelial cell migration (28). Blocking Ephrin A-1 with soluble EphA2-Fc receptor inhibited EphA2 signaling and decreased tumor-associated angiogenesis and, consequently, tumor progression (28, 29). Down-regulation of Ephrin A-1 by antisense RNA strategy decreased growth of colon cancer cells in three-dimensional cultures (22). Additionally, in vivo studies using antibody against EphA2 resulted in tumor regression in treated mice compared with controls (30). Together, these findings suggest that targeting Ephrin A-1 and EphA2 may be effective in inhibiting tumor progression and thereby provide a novel form of cancer therapy. Hence, it remains important to identify cancers that overexpress both Ephrin A-1 and EphA2.

Stable cell-cell contacts are necessary in enabling Ephrin A-1-EphA2 dimerization and, therefore, its biological function (12). Cell adhesion molecules such as E-cadherin maintain intimate cell-cell contacts. Indeed, the proper expression and function of the E-cadherin adhesion complex critically controls EphA2-Ephrin A-1 binding (31). In breast cancer cell lines, E-cadherin influenced the phosphorylation of EphA2 and its localization to the cell membrane (31). Moreover, E-cadherin has been implicated as a metastasis suppressor. For instance, loss of E-cadherin in cancer cell lines has been associated with loss of epithelial morphology and acquisition of tumor cell motility and invasion (32). In urinary bladder cancer, loss of E-cadherin has been associated with high-grade urothelial carcinomas, development of metastasis, and poor cancer prognosis (33, 34).

The expression and the relationships among EphA2, Ephrin A-1, and E-cadherin have not been reported in urinary bladder cancer. In this study, we determined the expression of EphA2, Ephrin A-1, and E-cadherin in cell lines and in clinical specimens from different stages of the bladder cancer. The phosphorylation status of EphA2 in bladder cancer cell lines was also assessed. Finally, we examined the potential effects of ligand stimulation of EphA2 on urinary bladder cancer cells using a novel adenoviral delivery system. These results suggest that EphA2 may provide a new and important target for clinical intervention against urinary bladder cancer.

Materials and Methods

Cell lines. Urinary bladder cancer cell lines RT4, T24, and UMUC-3 were obtained from American Type Culture Collection (Rockville, MD) specifically for these experiments. RT4 and T24 cell lines were cultured in McCoy’s 5A medium (Sigma, St. Louis, MO) with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and 1% L-glutamine. TCCSUP, HT1376, and UMUC-3 cells were cultured in MEM medium (Sigma) supplemented with 10% fetal bovine serum (Invitrogen) and 1% L-glutamine. All cell lines were grown at 37°C in the presence of 5% CO2.

Antibodies. Monoclonal antibody D7 against EphA2 was produced from hybridoma cultures in our laboratory as previously described (35). P-Tyr antibody (4G10) was purchased from Upstate Biologics, Inc. (Lake Placid, NY); EphA2 antibody from MedImmune (Gaithersburg, MD); β-catenin, β-actin, and horseradish peroxidase-Fc antibodies from Transduction Laboratories (Lexington, KY); Ephrin A-1 (sc-911) antibody from Santa Cruz Biotechnology (Santa Cruz, CA); and E-cadherin antibody from BD Biosciences Pharmingen (San Diego, CA).

Western blot analysis and immunoprecipitation. Cell lysates were prepared in 1% Triton lysis buffer (50 mmol/L Tris (pH 7.8), 150 mmol/L NaCl, 2 mmol/L EDTA, 1% Triton X-100) containing protease inhibitors (leupeptin, aprotinin, and Na-vanadate from Sigma). The cell lysates were stored at −80°C until further use. The protein concentration was determined using Coomassie reagent (Pierce, Rockford, IL). Equal amounts of proteins were size-fractionated on 7.5% SDS-PAGE gels, transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH) overnight, and the membranes were probed with antibodies as indicated in each experiment. The antibody-antigen binding complexes were detected by enhanced chemiluminescence (Pierce) and autoradiography using Kodak X-OMAT (Kodak, Rochester, NY). Blots were then stripped and probed with β-catenin or β-actin antibodies to confirm equal sample loading. For immunoprecipitation assays, cell lysates were incubated at 4°C for 1.5 hours with sepharose beads combined with rabbit anti-mouse immunoglobulin G and D7 antibody. The immunoprecipitates were washed several times in 1% Triton lysis buffer, resuspended in sample loading buffer, size-fractionated on SDS-PAGE gel, and analyzed with P-Tyr-specific antibody (4G10) or with D7 as a control for EphA2 levels.

Real-time reverse transcription-PCR analysis. The EphA2 mRNA levels in the bladder cancer cell lines (RT4, HT1376, T24, TCCSUP, and UMUC-3 cells) were measured by real-time reverse transcription-PCR. Total RNA was extracted from the cells using Trizol reagent (Invitrogen) as per instruction of the manufacturer. The cDNA pool for each cell line was synthesized using 1 μg of total RNA and SuperScript reverse transcriptase as described by the manufacturer (Clontech, Palo Alto, CA). For PCR analysis, primers for EphA2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were synthesized (IDT, Coralville, IA) and were as follows: EphA2 P1, 5′-ATGGAGCTCCAGGCGAGCCC-3′; EphA2 P2, 5′-GCCATACGGTCGTGTTGACCAGCCC-3′; GAPDH P1, 5′-CAGCTGTGACCTCGACGTTCGCT-3′; and GAPDH P2, 5′-CTCACGTGACCGAGGTATGCTGG-3′. A standard curve was obtained using known amounts of cDNA pool (Clontech). EphA2 and GAPDH genes were amplified from the cDNA pool using gene-specific primers and SYBR Green PCR core reagent (Perkin-Elmer Applied Biosystems, Foster City, CA) in a GeneAmp 5700 Sequence detection System (Perkin-Elmer Applied Biosystems). The PCR cycling conditions employed were as follows: 50°C/10 min, and 95°C/30 s, 60°C/1 min, 72°C/1 min for 40 cycles. One microliter of cDNA pooled from the bladder cancer cell lines indicated was amplified for EphA2 and GAPDH genes simultaneously as described above. The EphA2 PCR products were normalized to GAPDH PCR products and levels of...
EphA2 mRNA were determined. The relative amounts of EphA2 mRNA were calculated from the standard curve as directed (User Bulletin 2, Perkin-Elmer Applied Biosystems). Each sample was assayed in triplicates and the experiment was repeated twice.

**Tissue specimens.** Normal urothelium (n = 13) and carcinoma (n = 64) tissues from patients were obtained at the Indiana University School of Medicine with Institutional Review Board approval. Specimens were available from patients who had undergone transurethral cystoscopic biopsy or radical cystectomy as part of standard care of their condition. Tumor staging was reported at the time of diagnosis and followed the tumor-node-metastasis classification scheme (36, 37). Patients had received no radiation or chemotherapy before tissue collection. All specimens had been fixed in 10% formalin buffered saline and then embedded in paraffin.

**Immunohistochemistry.** Formalin-fixed, paraffin-embedded tissues were sectioned and immunostained by standard avidin-biotin-peroxidase complex method except that the antigen was retrieved from the deparaffinized, peroxidase blocked tissues using 0.2 mol/L Tris-HCl (pH 9.0) in a steam cooler for 20 minutes. The sections were incubated with EphA2 or Ephrin A-1 or E-cadherin antibodies followed by biotinylated horse anti-mouse or goat anti-rabbit (1:100 dilution; Vector Laboratories, Burlingame, CA). The signal was detected using 3,3′-diaminobenzidine reagent (DAKO Corporation, Carpenteria, CA), then counterstained with hematoxylin (Sigma), dehydrated, and mounted in permount (Sigma). Normal mouse serum or rabbit serum were substituted for the primary antibody as negative control and no detectable staining was observed. Specificity of EphA2 and Ephrin A-1 antibodies was detected by competition with EphA2 protein or with the peptide (Santa Cruz Biotechnology) used to raise Ephrin A-1 antibody, respectively. Positive controls for EphA2 and E-cadherin were breast tissue specimen and tonsil squamous epithelium (Lab Vision, Fremont, CA), respectively. All controls gave satisfactory results.

**Evaluation of immunostaining.** Two board-certified pathologists independently assessed the immunostained slides. Any difference in the immunohistochemical scores was resolved by consensus. The immunoreactivity of the cells was assessed for both the staining intensity and the percentage of tumor or normal cells stained. The staining intensity was scored on a scale of 1 to 4, with 1 being weakly immunoreactive and 4 being strongly immunoreactive.

**Statistical analysis.** The statistical analysis was done using Student’s t test, Tukey standardized test, and one-way ANOVA test. SAS software (Cary, NC) was used for the statistical analysis. P < 0.05 was considered significant in all analyses.

**Ephrin-A-1 (Ephrin A-1-Fc) treatment.** TCCSUP and T24 cells were plated at a density of 1 × 10^5 per well in six-well plates. Following overnight incubation at 37°C, Ephrin A-1-Fc at a concentration of 1 μg/ml was added to the cells. Whole-cell lysates were prepared as described above at intervals of 5, 15, 30, 45, 60 minutes, 2 and 4 hours, respectively, and fractionated on 7.5% SDS-PAGE gel. Western blots were analyzed with EphA2-specific antibody (D7) and detected using chemiluminescence. 12S-Actin antibody confirmed equal loading of proteins. The molecular weight markers were calculated from the standard curve as directed (User Bulletin 2, Perkin-Elmer Applied Biosystems). Each sample was assayed in triplicates and the experiment was repeated twice.

**Adenoviral delivery of Ephrin A-1-Fc.** Replication incompetent adenoviral vectors expressing the extracellular portion of Ephrin A-1 fused to Fc region of immunoglobulin (HAd-EA1Fc) and adenoviral parent vector (HAd-del E1E3) were produced as described (38) and infected into TCCSUP and RT4 cells. Cells were plated at a density of 7 × 10^3 per well in a 24-well plate and incubated at 37°C for 16 hours. Then media were replaced with 100 μl of PBS with Ca^2+ and Mg^2+ containing the viral particles at a multiplicity of infection of 10 plaque-forming units per cell and further incubated at 37°C for 30 minutes. The media were replenished and the plates were incubated at 37°C for 24, 48, 72, or 96 hours, respectively. At the end of each incubation period, the cell growth was assayed using Alamar blue reagent (Biosource International, Inc., Camarillo, CA) as described by the manufacturer. The reduction of Alamar blue was recorded at absorbance of 570 and 595 nm with percent reduction in Alamar blue being proportional to the number of cells. Western blot analysis was done with horseradish peroxidase-Fc to prove the production of Ephrin-A-1-Fc in the cell lysates and in the conditioned medium obtained following adenoviral infection. A band of ~66-kDa was detected in Western blots.

**Results**

**Expression of EphA2 protein in bladder cancer cell lines.** Our initial studies of EphA2 in bladder cancer used cell models of the disease such as a low-grade papillary tumor cell line (RT4) and cell lines derived from high-grade invasive bladder cancer (T24, HT1376, UMUC-3, and TCCSUP; ref. 39). Figure 1A shows expression levels of EphA2 in these cell lines. RT4 cells expressed low levels of EphA2 protein whereas HT1376 cells had modest levels of the protein. Higher expression of EphA2 protein was detected in T24, TCCSUP, and UMUC-3.

![Figure 1A](https://www.aacrjournals.org)
Expression of EphA2 mRNA levels in bladder cancer cell lines. To investigate the transcription-translation of EphA2, we measured EphA2 mRNA levels in the bladder cancer cell lines (RT4, HT1376, T24, TCCSUP, and UMUC-3 cells) by real-time reverse transcription-PCR as shown in Fig. 1C. The EphA2 PCR products were normalized to GAPDH PCR products and levels of EphA2 mRNA were determined. The EphA2 mRNA levels in RT4, HT1376, T24, TCCSUP, and UMUC-3 cells were 0.8, 3.1, 1.53, 3.65, and 1.35 units, respectively. On comparing the levels of EphA2 protein (Fig. 1B) to those of EphA2 mRNA, the low levels of EphA2 protein in RT4 cells related to low levels of EphA2 mRNA. In contrast, the levels of EphA2 protein in all other cell lines indicated were disproportionate with what might have been predicted by mRNA expression. These results suggest that posttranscriptional regulatory mechanisms also contribute to the high levels of EphA2 protein in bladder tumor cells.

Expression of EphA2 in normal and carcinoma tissues. In normal and carcinoma bladder tissues, 80% to 90% of urothelial cells had some EphA2 immunoreactivity (Fig. 2A-C). The immunoreactivity was distributed diffusely throughout the cytoplasm in the tumor cells. EphA2 immunoreactivity was uniform throughout each specimen, with little evidence of heterogeneity in EphA2 levels between different tumor cells. The intracellular distribution of EphA2 was similarly diffuse, with both cytoplasmic and membrane staining observed. Notably, EphA2 immunoreactivity was restricted to bladder carcinoma cells, with no staining of connective tissues. The specificity of EphA2 staining was confirmed by competition with EphA2-Fc protein (data not shown). In normal tissues, 85% of the samples had weakly positive stained cells (score of 1) and the rest of the samples had moderately positive stained cells (score of 2). The staining intensity of EphA2 was significantly higher ($P < 0.0001$) in carcinoma (for each $T_1$, $T_2$, $T_3$, or $T_4$ stage and collectively) than in normal tissues. Within the carcinoma samples, there was an increase in immunoreactivity with increasing stages of the bladder carcinoma (Fig. 3A). In $T_1$ lesions, 30% of tumor specimens had a staining intensity of 3 and none had an intensity of 4. In $T_3$ and $T_4$ carcinomas, 75% to 90% of the tumor samples had staining intensity of 3 to 4, as shown in Fig. 3B. The EphA2 staining intensity was significantly greater in $T_3$-$T_4$ stages than in $T_1$ stage of the bladder cancer ($P < 0.0001$).

Expression of Ephrin A-1 in normal and carcinoma tissues. In normal urothelium, immunoreactivity to Ephrin A-1 was noted in 80% to 90% of urothelial cells with staining intensity of 2 in the majority (85%) of the tissues. Ephrin A-1 immunoreactivity was observed in the cell membrane and in the cytoplasm with very minimal staining of the surrounding connective tissue as shown in Fig. 2D. In all carcinoma tissues, 80% to 90% of the cells had Ephrin A-1 immunoreactivity, and the staining intensity was 3 to 4 in most sections (Fig. 3C). The staining was...
distributed throughout the cell cytoplasm as shown in Fig. 2E and F. The most intense staining (score of 4) was observed in 25% to 40% of carcinoma sections, including early and advanced stages, as shown in Fig. 3C. Competition experiments with the immunogenic peptide confirmed the specificity of Ephrin A-1 immunoreactivity (data not shown). When compared with normal tissue sections, Ephrin A-1 staining intensity was significantly greater in all stages (T<sub>a</sub>, P < 0.0001; T<sub>1</sub>, P < 0.0001; T<sub>2</sub>, P < 0.0158; T<sub>3</sub>, P < 0.0001; and T<sub>4</sub>, P < 0.0001) of bladder cancer.

Expression of E-cadherin in normal and carcinoma tissues. Based on the evidence linking the functions of EphA2, Ephrin A-1, and E-cadherin, E-cadherin immunoreactivity was also evaluated in the same tissue specimens (Fig. 2G-I). In normal urothelium, E-cadherin immunoreactivity was limited to the cell membrane, and the tissues analyzed had strong positive cell membrane staining. In carcinoma tissues, E-cadherin staining was generally increased as bladder cancer advances. E-cadherin immunoreactivity in carcinoma tissues, when observed in both the cell membrane and the cytoplasm with a staining intensity of 2 to 3 in most cases (Figs. 3A and 2H and I). The staining intensity was significantly lower in T<sub>2</sub> (P < 0.02), T<sub>3</sub> (P < 0.0001), and T<sub>4</sub> (P < 0.002) stages than in normal tissues. In contrast, the pattern and intensity levels of E-cadherin in early-stage cancer (T<sub>a</sub> and T<sub>1</sub>) did not differ significantly from those observed in normal tissues (P < 0.070 and P < 0.079, respectively; Fig. 3D).

Association between EphA2 and Ephrin A-1 expression, as well as between EphA2 and E-cadherin expression, and stage of bladder cancer. When evaluated independently, the levels of staining intensity of EphA2 or Ephrin A-1 were significantly increased in carcinoma compared with those of normal tissues. When considered together, their expressions were significantly higher in T<sub>1</sub>-T<sub>2</sub> stages (P < 0.0399) and T<sub>3</sub>-T<sub>4</sub> stages (P < 0.0001) compared with T<sub>a</sub> stage of bladder cancer. In addition, an inverse association between E-cadherin and EphA2 was noted with increasing stage of bladder carcinoma. Similarly, when EphA2 (increased) and E-cadherin (decreased) were considered together, there was a significant relationship between T<sub>1</sub>-T<sub>2</sub> stages (P < 0.00001) compared with T<sub>a</sub> stage. This relationship did not hold true when comparing T<sub>1</sub>-T<sub>2</sub> to T<sub>a</sub> stage. Notably, EphA2 expression increased significantly (3-fold) in early stage of bladder cancer whereas the E-cadherin levels decreased (1.2-fold) compared with normal bladder tissue.

EphA2 stimulation in bladder cancer cells. The inverse relationship between EphA2 and E-cadherin in clinical specimens was intriguing in light of the evidence that improper functioning of E-cadherin decreases ligand binding. We therefore determined the expression pattern of E-cadherin in the bladder cancer cell lines. High E-cadherin expression was observed in RT4 and HT1376 cell lines that have low to moderate amounts of EphA2, whereas in T24 and UMUC-3 cells that have very high expression of EphA2, no E-cadherin expression was detected and TCCSUP showed very low expression (Fig. 4A). In other tumor types, this defect prevents tyrosine phosphorylation of EphA2 and thereby causes EphA2 to accumulate in tumor cells and increases EphA2 oncogenic activity (31). To examine the potential relevance of this hypothesis to bladder cancer, the EphA2 in the different bladder carcinoma-derived cells was isolated by immunoprecipitation and the precipitant was subjected to Western blot analyses with phosphotyrosine-specific antibodies. In RT4 and HT1376 cells, phosphorylation of EphA2 was observed although these cells had relatively low levels of EphA2 protein (Fig. 4B). The phosphotyrosine content of EphA2 was undetectable in those tumor cells that had the highest expression of EphA2 (T24, TCCSUP, and UMUC-3). Probing the same blot with EphA2-specific antibodies (D7) confirmed the presence of EphA2 in these cell lines (Fig. 4B (B)).

EphA2 protein in bladder cancer cell models is degraded by ligand Ephrin A-1. In light of the decreased ligand binding in malignant bladder cells, we then asked if these cells would
respond to restoration of ligand binding. Our initial studies evaluated the effects of an artificial ligand (Ephrin A-1-Fc) which does not require proper E-cadherin function to stimulate EphA2. Western blot analyses revealed that Ephrin A-1-Fc treatment of TCCSUP or T24 cells induced EphA2 stimulation and subsequent protein degradation (Fig. 5 and data not shown). To confirm equal sample loading, the membranes were stripped and reprobed with antibodies specific for β-catenin (Fig. 5A, bottom). This decrease in EphA2 protein was associated with phosphorylation of EphA2, as shown in Fig. 5B.

We also investigated the effect of Ephrin A-1 on E-cadherin levels following artificial ligand stimulation of TCCSUP cells and found no difference in E-cadherin levels by Western blot analysis (data not shown), suggesting that artificial ligand is more directed towards EphA2 function. Similarly, we then asked if persistent stimulation of Ephrin A-1-Fc could affect E-cadherin levels. We observed no changes in E-cadherin levels of adenovirus treated with Ephrin A-1-Fc as compared with parent control virus and uninfected TCCSUP cells (data not shown). This suggests that Ephrin A-1 does not affect E-cadherin levels, but change in the E-cadherin level is a primary event that disrupts cell-cell contact and prevents EphA2-Ephrin A-1 interaction.

**Treatment with extracellular domain of Ephrin A-1 decreases cell growth.** Previous studies indicate that Ephrin-A-1-Fc is relatively unstable and thus can lead to relatively short-term effects on tumor cell behavior. To extend these findings further, we were able to achieve persistent stimulation of EphA2 by encoding Ephrin A-1-Fc in adenoviral vectors. A human adenoviral vector that expresses Ephrin A-1-Fc (HAD-Ephrin A-1F-c) was used to infect TCCSUP and RT4 cells. Western blot analyses of cell supernatants confirmed that the infected cultures secreted Ephrin A-1-Fc (Fig. 6A-C). Cells transfected with parent adenoviral vector (HAd-del E1E3) were used to detect any nonspecific effects of growth inhibition from adenovirus itself and taken as control (100%). Adenoviral delivery of Ephrin A-1 to bladder cancer cells (TCCSUP) resulted in 71%, 70%, 68%, and 46% cell growth at 24, 48, 72, and 96 hours, respectively, compared with parent adenoviral vector. In RT4 cells that have relatively low EphA2 expression, no reduction in cell growth was observed following adenoviral infection as compared with controls (Fig. 6D).

![Fig. 5. A, degradation of EphA2 protein with Ephrin A-1 stimulation. TCCSUP cells were incubated in the presence of 1 μg/mL Ephrin A-1-Fc at 37°C for 0 minutes to 4 hours. Twenty-five micrograms of protein from each cell lysate were fractionated on SDS-PAGE. Western blot analysis was carried out with the EphA2-specific antibody D7 and detected using chemiluminescence. β-Catenin antibody confirmed equal sample loading. Molecular weight markers are indicated on the left. B, degradation of EphA2 occurs via phosphorylation. TCCSUP cells were incubated in the presence of 1 μg/mL Ephrin A-1-Fc at 37°C for 0 minutes to 4 hours. Immunoprecipitates of EphA2 from cell lysates were fractionated on SDS-PAGE. Western blot analysis was done using p-Tyr antibody (4G10, A) and detected using chemiluminescence kit. Duplicate cell lysates were resolved simultaneously on the same gel and probed with EphA2-Fc antibody (D7, B). Sepharose beads linked to immunoglobulin G were included as negative control.](image_url)

![Fig. 4. A, E-cadherin expression in bladder cancer cell lines. Twenty-five micrograms of protein from whole-cell lysates were fractionated on 7.5% SDS-PAGE gel. The blot was probed with E-cadherin antibody, then stripped and reprobed with β-actin antibody for loading control. B, EphA2 phosphorylation in bladder cancer cells. EphA2 protein was immunoprecipitated from 100 μg of whole-cell lysates and resolved by SDS-PAGE. The blots were probed with p-Tyr antibody-4G10 (top) and detected by chemiluminescence. Duplicate cell lysates were resolved simultaneously on the same gel and probed with EphA2-specific antibody (D7, bottom). EphA2 protein level from each cell lysate is indicated. TCCSUP treated with Ephrin A-1-Fc was the positive control and sepharose beads linked to immunoglobulin G were the negative control. Molecular weight markers are indicated on the left.](image_url)

**Discussion**

A major finding of this study was the overexpression of EphA2 in human urothelial carcinoma. EphA2 was identified in urothelial carcinoma cell lines by Western blot, with high expression noted in three of the five cell lines tested. Similarly, immunohistochemistry analyses for EphA2 showed much higher staining intensity in urothelial carcinoma than in normal tissues and a significant increased expression with increasing stage of the disease. An inverse association between E-cadherin and EphA2 expression was noted. In three of the four cell lines derived from invasive urothelial carcinoma, EphA2 was not phosphorylated. Ligand binding resulted in the induction of phosphorylation and degradation of EphA2 protein. This study also provided evidence that urothelial carcinoma growth can be inhibited by therapies that target EphA2 ligand binding. Adenoviral delivery of Ephrin A-1-Fc resulted in a decrease in proliferation of bladder cancer cells. Together, these data show that...
EphA2 is overexpressed in urinary bladder cancer and suggest that EphA2 can be a new therapeutic target in bladder cancer treatment. Identifying expression of EphA2 is important as new therapies that target this molecule are emerging. EphA2 antibody treatment of athymic mice bearing MDA231 xenografts was sufficient to cause tumor regression with no adverse toxicity (30). This provides further support for evaluating therapies that target EphA2 in other cancers, including urinary bladder cancer.

In this study, Ephrin A-1 expression was also found to be greater in urothelial carcinoma than in normal tissues. Ephrin A-1 was present at similar levels across all stages of bladder cancer. In bladder cancer cell lines, Ephrin A-1 binding to EphA2 protein caused EphA2 phosphorylation and degradation (Fig. 5A and B). This raises the question of why EphA2 persists in urothelial carcinoma tissues and why Ephrin A-1 expression with a concomitant decrease in E-cadherin expression was observed (Figs. 2 and 3) in more advanced stages of bladder cancer (T3–T4). This finding is similar to that reported in colorectal cancer wherein EphA2 expression was inversely correlated to E-cadherin expression (42).

Although EphA2 was overexpressed in carcinoma, it is likely that it did not interact with its ligand, Ephrin A-1. Appropriately this seeming paradox reflects experimental observations that the proper functioning and expression of the E-cadherin cell-cell adhesion protein is necessary to stabilize EphA2-Ephrin A-1 binding. Our present findings indicate that the levels and membrane localization of E-cadherin decrease in malignant carcinoma specimens. Thus, the corresponding increases in EphA2, as well as its cytoplasmic diffuse localization, may reflect these changes in E-cadherin. Nonphosphorylated EphA2 can promote metastasis in laboratory settings and high levels of EphA2 relate to metastatic disease and decreased survival in other cancer types (20, 24). Thus, future studies should evaluate whether the expression or phosphotyrosine content of EphA2 in clinical bladder cancer specimens relates to E-cadherin and metastatic potential.

Another novel outcome of our present study is that adenoviral delivery of ligands (Ephrin A-1) to the EphA2 on tumor cells is sufficient to decrease bladder cancer cell growth. In fact, adenoviral-Ephrin A-1 inhibited growth of bladder cancer cells in vitro by >50% (Fig. 6D). This further suggests that EphA2 can be down-regulated or degraded and that EphA2 may be an important target in bladder cancer treatment. Consistent with this finding, ligand binding has been reported to reverse the malignant behavior of breast cancer cells in vitro and in vivo (43). Indeed, the use of agonist antibodies is the subject of extensive preclinical investigation and will soon enter clinical trials. However, to our knowledge, the present findings are the first to apply an agonist-restoration strategy to bladder cancer. Other approaches to target Eph signaling include the use of agents that interfere with ligand binding although these approaches have been largely restricted to intervention against EphA2 on angiogenic blood vessels (28).

In conclusion, the results of this study show the expression of EphA2 and Ephrin A-1 in urinary bladder cancer. EphA2 protein in bladder cancer cells is not phosphorylated, which is consistent with recent reports that unphosphorylated EphA2 favors the aggressive phenotype of cancer cells. Moreover, these results show the potential for using an agonist to EphA2 to treat urinary bladder cancer.
References

Expression of EphA2 and Ephrin A-1 in Carcinoma of the Urinary Bladder

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