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Low Expression of EphB2, EphB3, and EphB4 in Bladder Cancer: Novel Potential Indicators of Muscular Invasion

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Purpose: Eph receptors are differentially expressed in numerous malignant tumors. This study intended to analyze the roles of EphB receptors (EphB2, B3, and B4) in urinary bladder cancer.
Materials and Methods: Tissue microarray-based immunohistochemical analysis was used to investigate the expression patterns of EphB2, EphB3, and EphB4 in 154 bladder cancer specimens. Immunohistochemical staining was conducted examining the extent of stained cells and staining intensity. EphB was considered to be highly expressed when the intensity of staining was more than moderate in >25% of cells in the tissue section. Small interfering RNA (siRNA) was used to knock down EphB expression in bladder cancer cell lines (T24, 5637) to determine the effects of EphB on tumor cell invasion, proliferation, and migration.
Results: EphB receptors (B2, B3, and B4) were detected in 40.9% (EphB2, 63/154), 71.4% (EphB3, 110/154), and 53.2% (EphB4, 82/154) of bladder cancer specimens. Low expression of EphB2, B3, and B4 receptors were significantly associated with higher tumor grade (EphB2, $p<0.001$; EphB3, $p=0.032$; EphB4, $p<0.001$) and muscular invasion (EphB2, $p=0.002$; EphB3, $p=0.009$; EphB4, $p<0.001$). No obvious correlation was observed with other clinicopathological variables, such as age, sex, recurrence, lymph node involvement, metastasis, and overall survival. Inactivation of EphB receptors by siRNA transfection increased cell viability, tumor cell invasion, proliferation, and migration in comparison with untransfected cancer cells.
Conclusion: Low expression of EphB receptors (B2, B3, and B4) can be a predictive marker for muscular invasion of bladder cancer.

Key Words: EphB2, EphB3, EphB4, bladder cancer, muscle invasion, predictive marker

INTRODUCTION

Urinary bladder cancer is the most lethal urologic malignancy worldwide. It is estimated to affect nearly 75000 people in the United States, with 15000 people dying from the disease every year.[1] About 75% of bladder cancer patients present with non-muscle-invasive bladder cancer, with the remaining presenting with muscle-invasive bladder cancer, including metastatic cancer. Non-muscle-invasive bladder cancer patients are initially treated with transurethral resection and half of these patients experience recurrence of the disease. About 5–30% of these patients progress to muscle-invasive bladder cancer.[2] In spite of an overall 5-year survival rate for bladder cancer of 82%, the 5-year survival rate for metastatic cancer only 6%: the 5-year survival rate for localized cancers is 94%.[3] Since most initial diagnoses are made from transurethral bladder biopsies, it can difficult to determine the tumor is invasive. Therefore, there is a clear need for both predictors of muscular invasion and effective targets for novel systemic therapies.

Eph/ephrin signaling mediates angiogenesis and has been shown to be associated with regulation of cell growth, morphology, migration, and survival. Additionally, Eph/ephrin signaling has also been found to be associated with the regulation of cytoskeleton organization.[4] Eph receptors are differentially expressed in various cancers, promoting cancer growth or sup-

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pressing cancer progression depending on the cancer type.[5] EphB expression has been shown to be related to poor prognosis in osteosarcoma, endometrial cancer, and melanoma.[6,7]

A retrospective study indicated that EphB4, EphB6, EphA2, EphA4, and EphA7 were significantly associated with poor overall survival in breast cancer patients.[8] Their results demonstrated that several Eph receptors may be important targets for treatment in breast cancer. Another study demonstrated that EphB4 was overexpressed in bladder cancer cells and mediated cell survival.[9] Additionally, several studies have demonstrated that EphB4 is overexpressed in a few different cancers, including head and neck, breast, prostate, and uterine cancer.[10-13] In breast cancer, Eph receptor expression was found to be significantly associated with cancer aggressiveness and invasiveness. In an animal model of breast cancer, overexpression of EphA2 induced tumor progression and promoted metastasis.[14] Notwithstanding, the clinical significance of EphB expression in cancer is controversial. Loss of EphB2 expression has been shown to be correlated with more advanced colorectal cancer and poorer overall survival.[15] In bladder cancer, researchers indicated that loss of EphB2 expression and overexpression of EphB4 could possibly affect tumor progression.[16] Meanwhile, EphB3 knockout mice exhibited an increased frequency of retinal ganglion cell axon guidance errors to the optic disc, where EphB3 is involved in the formation of topographic maps.[17] However, in malignant tumors, the function of EphB3 remains unknown. Moreover, while some researchers have reported a suppressive effect for EphB receptors (B2, B3, and B4) in colorectal cancer,[18] other noted tumor-promoting properties for EphB3 in lung cancer.[19]

The Eph family comprises a group of receptor protein tyrosine kinases that are involved in parenchyma tissue integrity and cell-cell communication during embryogenesis. Since the disorganization of tissue architecture is critical for tumor invasiveness, we aimed to examine the expression of EphB family receptors during bladder cancer progression. To our knowledge, EphB (B2, B3, and B4) expression in urinary bladder cancer has not been well investigated, and to elucidate the roles of EphB receptors in bladder cancer, we examined the expression of EphB receptors in surgical bladder specimens and performed functional studies using bladder cancer cell lines.

MATERIALS AND METHODS

Patients and tissue samples for EphB (B2, B3, and B4) immunohistochemistry

Cases of bladder cancer treated at the CHA Bundang Medical Center, School of Medicine, CHA University from 2006 to 2012 were reviewed. A total of 154 specimens of bladder cancer were obtained from 134 patients who underwent transurethral resection of the bladder (TURB) or partial cystectomy or radical cystectomy. Eighteen patients had repeated surgery (6 patients: TURB and repeated TURB; 7 patients: TURB and radical cystectomy; 3 patients: TURB and partial cystectomy; 1 patient: TURB and repeated TURB twice; 1 patient: two times of TURB and radical cystectomy). When possible, adjacent normal tissue was also collected. Human specimens were collected under approval from the CHA Bundang Medical Center Institutional Review Board (IRB2017-09-052).

Analysis and grading of tissues were performed by a blinded reviewer. A manual tissue arrayer (Quick-Ray Manual Tissue Microarrayer; Unitma Co. Ltd., Seoul, Korea) was used to construct issue microarrays from archival formalin-fixed, paraffin-embedded tissue blocks. Tissue cylinders (diameter: 3 mm) were punched from the tumor region of the donor block and were re-embedded into the recipient block. Four μm sections were made from tissue microarrays.

Immunohistochemistry

Tissue microarray sections were deparaffinized in xylene and rehydrated in alcohol. Endogenous peroxidase activity was suppressed by 10 min of immersion in 3% hydrogen peroxide. For antigen retrieval, each section was heated in 0.01 mol/L sodium citrate buffer (pH 6.0) for 30 min. The sections were washed three times in phosphate-buffered saline (PBS) for 5 min and incubated for 1 h at room temperature with mouse monoclonal antibodies to human EphB2, EphB3, and EphB4 (1:100; Epitomics, Burlingame, CA, USA). The sections were then incubated with horseradish peroxidase-labeled rabbit anti-mouse immunoglobulin (DAKO; 1 h at room temperature) and developed with diaminobenzidine at room temperature to reveal staining.

Interpretation of immunostaining

Positive ephrin receptor (EphB2, B3, and B4) staining was defined as brown granules in the cytoplasm or nuclei. Staining intensity was evaluated as no staining, weak intensity, moderate intensity, or strong intensity. The extent of staining was categorized as follows: 0–4%, 5–25%, 26–50%, 51–75%, and 76–100%. A case was considered to highly express EphB when the intensity of staining was more than moderate in >25% of cells in the tissue section.

Cell lines and culture

The human bladder cancer cell lines (T24, 5637) were obtained from the American Type Culture Collection (Rockville, MD, USA). T24 and 5637 cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin, and incubated at 37°C in a humidified atmosphere consisting of 5% CO_2.

EphB siRNA transfection

The siRNA used to silence EphB (B2, B3, and B4) and the negative control vector (cat. no. SN-1013) were purchased from Bi-

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oneer, Inc. (Daejeon, Korea) Cells were plated at 70% confluency in RPMI-1640 containing 10% serum without antibiotics. The EphB (B2, B3, and B4) siRNA and control vector were transfected into the cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), and the cells were assayed at 24, 48, and 72 h after transfection. The siRNA sequences were as follows: si-EphB2, sense: 5'-GAG AAG UUG CCA CUC AUC A(dTdT)-3' and antisense: 5'-UGA UGA GUG GCA ACU UCU C(dTdT)-3'; si-EphB3, sense: 5'-GGA CUU UCG GAC UCU UGG A(dTdT)-3' and antisense: 5'-UCC AAG AGU CCG AAA GUC C(dTdT)-3'; si-EphB4, sense: 5'-GUC AUG AUU CUC ACA GAG U(dTdT)-3' and antisense: 5'-ACU CUG UGA GAA UCA UGA C(dTdT)-3'.

Quantitative real-time PCR for Eph receptors (EphB2, B3, and B4)

Total RNA was extracted from cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. For first-strand cDNA synthesis, 1 μg of total RNA was reverse-transcribed in a 20 μL reaction mix using a Superscript III kit (Invitrogen). Quantitative real-time PCR was performed in triplicate using the Bio-Rad CFX96 Real-Time PCR Detection System. TaqMan Gene Expression Assay (Applied Biosystems, Paisley, UK) was used to analyze GAPDH (ABI code: Hs02758991_gL, sequence undisclosed), EphB2 (ABI code: Hs00362096_mL, sequence undisclosed), EphB3 (ABI code: Hs00177903_mL, sequence undisclosed), and EphB4 (ABI code: Hs01119113_mL, sequence undisclosed). The final reaction mix volume of 20 μL included 1-μL cDNA, 10 μL of TaqMan master mix (Applied Biosystems), and 1 μL primer and probe kit (Applied Biosystems). The reverse transcription conditions used were 2 min at 50°C, 10 min at 95°C, and 1 min at 60°C for 40 cycles. Transcript levels were normalized against GAPDH expression. Gene expression was calculated using $2^{-\Delta\Delta Ct}$.[20]

Western blot analysis

Cells were lysed with Protein Extraction Reagent (Pro-Prep, iNtRON Biotechnology, Seongnam, Korea) and the lysates were centrifuged at 13000 rpm at 4°C for 15 min. Protein concentrations were determined using the Bradford assay. Equal amounts of proteins were electrophoresed on 10% SDS-PAGE and transferred to nitrocellulose membranes at 100 V for 2 h. The membranes were blocked with 5% milk for 1 h. The membranes were incubated overnight at 4°C with 1:1000 dilution of anti-EphB2 (Cell Signaling Technology, Danvers, MA, USA), anti-EphB3 (Thermo Fisher Scientific, Inc., Waltham, MA, USA), anti-EphB4 (Cell Signaling Technology), and 1:10000 dilution of anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Then, the membranes were incubated with anti-rabbit 1:5000 or anti-mouse 1:5000 (Santa Cruz Biotechnology) secondary antibodies for 1 h at room temperature. After incubation, the protein bands were observed using ECL reagent (iNtRON Biotechnology). Protein expression was quantified using Quantity One® 1-D Analysis Software (Bio-Rad Laboratories, Hercules, CA, USA).

Wound-healing assay

A wound-healing assay was used to assess cell migration. Cells were seeded into 96-well tissue culture plates and cultured to a confluent monolayer. A sterile pipette tip was used to scratch the wound carefully. The monolayer was incubated in cell culture medium and then imaged at 24 h using a microscope.

Colony-forming assay

T24 and 5637 cells (1×10^5/well) were seeded in six-well plates. The next day, cells were transfected with EphB2, EphB3, and EphB4 siRNA and incubated for 48 h. Then, transfected cells were replated at 300 cells/well in six-well culture dishes. After 10 days, colonies were fixed with 4% paraformaldehyde for 10 min and visualized using hematoxylin. The number of colonies was defined as >50 cells/colony and counted.

Cell invasion assay

Matrigel invasion assay was performed using Boyden chambers containing Transwell membrane, which was coated with BD Matrigel (BD Biocoat, Bedford, MA, USA). 1×10^4 cells in McCoy's 5A medium supplemented with 0.1% BSA were plated in the upper chamber. McCoy's 5A medium containing 10% FBS was added to the lower chamber. After 48 h of incubation, the non-invasive cells on the upper surface of the membrane were eliminated with a cotton swab. The cells that had passed to the lower surface of the membrane were fixed in 100% ethanol and stained with hematoxylin and eosin.

Statistical analysis

Statistical analysis was performed using SPSS 24.0 software (IBM Corp., Armonk, NY, USA). The χ^2 test and Kruskal-Wallis test were performed to assess associations between variables. For survival analysis, the Kaplan-Meier method and log-rank test were used. A one-way analysis of variance was used to analyze colony forming, invasion, and wound healing assays. A p-value<0.05 was considered statistically significant.

RESULTS

Association between EphB (B2, B3, and B4) immunoreactivity and clinicopathologic parameters in patients with bladder cancer

To investigate whether EphB (B2, B3, and B4) expression is associated with bladder cancer, we analyzed EphB expression in 154 bladder cancer tissues. Of 154 specimens, 117 specimens were obtained from TURB, 14 specimens from partial cystectomy, and 23 specimens from radical cystectomy. Representative immunoreactivity of EphB (B2, B3, and B4) expression is shown in Fig. 1. EphB (B2, B3, and B4) expression revealed strong membranous staining, mostly combined with a cyto-

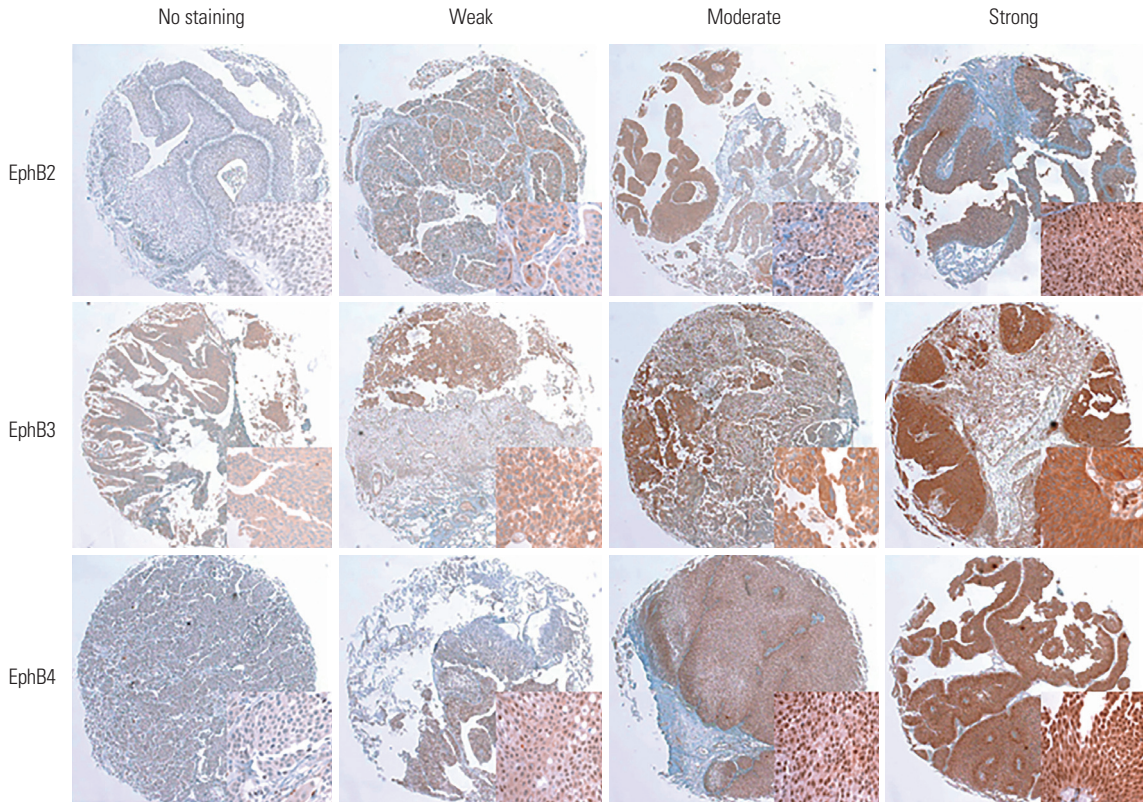

Fig. 1. Representative immunoreactivity of EphB expression. EphB receptors (B2, B3, and B4) were detected in 40.9% (EphB2, 63/154), 71.4% (EphB3, 110/154), and 53.2% (EphB4, 82/154) of the bladder cancer specimens (Inlet: high magnification, ×400).

plasmic immunostaining pattern tumor cells. EphB receptors (B2, B3, and B4) were detected in 40.9% (EphB2, 63/154), 71.4% (EphB3, 110/154), and 53.2% (EphB4, 82/154) of the bladder cancer tissues. Low expression of EphB2, B3, B4 receptors were significantly associated with higher tumor grade (EphB2, $p<0.001$; EphB3, $p=0.032$; EphB4, $p<0.001$) and muscular invasion (EphB2, $p=0.002$; EphB3, $p=0.009$; EphB4, $p<0.001$) (Table 1). Low expression of EphB2 and EphB4 were significantly associated with high clinical stage (EphB2, $p=0.004$; EphB4, $p<0.001$). Similarly, patients with low expression of EphB2 and EphB4 were significantly more likely to undergo partial or radical cystectomy (EphB2, $p=0.002$; EphB4, $p<0.001$). Fig. 2 depicts a significant association between the low expression of EphB (B2, B3, and B4) receptors and muscular invasiveness. Interestingly, high EphB (B2, B3, and B4) expression groups exhibited a correlation with non-muscular invasion of cancer. No obvious correlation was observed with other clinicopathological variables, such as age, sex, recurrence, lymph node involvement, and metastasis. There was no significant association between EphB (B2, B3, and B4) expression and overall survival (Fig. 3).

EphB siRNA inhibits EphB expression in bladder cancer cell lines

We employed siRNA against EphB (B2, B3, and B4) via transfection into T24 and 5637 bladder cancer cells. We examined EphB (B2, B3, and B4) mRNA expression in comparison with control cells after transfection at 24, 48, and 72 h, respectively. The results showed that EphB2 mRNA expression in T24 (72%, 50%, and 30.4%) and 5637 (61.6%, 73.6%, and 35.1%) cells was decreased ($p<0.05$) (Fig. 4A), as was EphB3 mRNA expression in T24 (72%, 42%, and 17%) and 5637 (53%, 62.2%, and 60%) cells ($p<0.05$) (Fig. 4B) and EphB4 mRNA expression in T24 (61.5%, 81.6%, and 50.6%) and 5637 (76.5%, 61.2%, and 43.5%) cells ($p<0.05$) (Fig. 4C). These results confirmed the effective suppressive effect of EphB (B2, B3, and B4) siRNA.

To demonstrate the efficiency of EphB (B2, B3, and B4) silencing at the protein level, we employed Western blot analysis to determine EphB (B2, B3, and B4) protein expression levels at 24, 48, and 72 h after transfection. We discovered that EphB (B2, B3, and B4) expression in T24 (51%, 69%, and 45%; 51%, 80%, and 95%; 86%, 62%, and 44%, respectively) and 5637 (53%, 62%, and 27%; 48%, 71%, and 92%; 84%, 46%, and 53%, respectively) cells decreased in comparison with the control cell line ($p<0.05$) (Fig. 5).

Effect of EphB suppression on cancer cell proliferation, migration, and invasiveness in EphB siRNA transfected bladder cancer cell lines

We conducted colony-forming, wound healing, and Matrigel invasion assays after EphB (B2, B3, and B4) siRNA transfection. Colony numbers for EphB (B2, B3, and B4) transfected

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Table 1. Correlations among EphB Expression and Clinicopathological Parameters of Bladder Cancer

	Total (n=154)	EphB2		*p* value	EphB3		*p* value	EphB4		*p* value
		Low	High		Low	High		Low	High	
Age (yr)										
<60	41 (27)	20 (48.8)	21 (51.2)		8 (19.5)	33 (80.5)		16 (39.0)	25 (61.0)	
≥60	113 (73)	71 (62.8)	42 (37.2)	0.117	36 (31.9)	77 (68.1)	0.134	56 (49.6)	57 (50.4)	0.247
Sex										
Male	118 (77)	71 (60.2)	47 (39.8)		36 (30.5)	82 (69.5)		55 (46.6)	63 (53.4)	
Female	36 (23)	20 (55.6)	16 (44.4)	0.622	8 (22.2)	28 (77.8)	0.335	17 (47.2)	19 (52.8)	0.949
Tumor grade										
Low	59 (38)	24 (40.7)	35 (59.3)		11 (18.6)	48 (81.4)		15 (25.4)	44 (74.6)	
High	95 (62)	67 (70.5)	28 (29.5)	<0.001*	33 (34.7)	62 (65.3)	0.032*	57 (60.0)	38 (40.0)	<0.001*
TNM stage										
Stage 0a/0is	64 (41)	26 (40.6)	38 (59.4)		11 (17.2)	53 (82.8)		16 (25.0)	48 (75.0)	
Stage I	21 (14)	15 (71.4)	6 (28.6)	0.004*†	6 (28.6)	15 (71.4)	0.076†	18 (38.1)	13 (61.9)	<0.001*†
Stage II	38 (25)	27 (71.1)	11 (28.9)		14 (36.8)	24 (63.2)		25 (65.8)	13 (34.2)	
Stage III	21 (14)	15 (71.4)	6 (28.6)		8 (38.1)	13 (61.9)		17 (81.0)	4 (19.0)	
Stage IV	10 (6)	8 (80.0)	2 (20.0)		5 (50.0)	5 (50.0)		6 (60.0)	4 (40.0)	
Muscular invasion										
No	85 (55)	41 (48.2)	44 (51.8)		17 (20.0)	68 (80.0)		24 (28.2)	61 (71.8)	
Yes	69 (45)	50 (72.5)	19 (27.5)	0.002*	27 (39.1)	42 (60.9)	0.009*	48 (69.6)	21 (30.4)	<0.001*
Recurrence										
No	108 (70)	65 (60.2)	43 (39.8)		32 (29.6)	76 (70.4)		50 (46.3)	58 (53.7)	
Yes	46 (30)	26 (56.5)	20 (43.5)	0.672	12 (26.1)	34 (73.9)	0.656	22 (47.8)	24 (52.2)	0.862
LN involvement										
No	150 (97)	88 (58.7)	62 (41.3)		42 (28.0)	108 (72.0)		71 (47.3)	79 (52.7)	
Yes	4 (3)	3 (75.0)	1 (25.0)	0.512	2 (50.0)	2 (50.0)	0.336	1 (25.0)	3 (75.0)	0.377
Metastasis										
No	149 (97)	87 (58.4)	62 (41.6)		41 (27.5)	108 (72.5)		69 (46.3)	80 (53.7)	
Yes	5 (3)	4 (80.0)	1 (20.0)	0.334	3 (60.0)	2 (40.0)	0.114	3 (60.0)	2 (40.0)	0.546
Surgical procedure										
TURB	117 (76)	61 (52.1)	56 (47.9)		30 (25.6)	87 (74.4)		43 (36.8)	74 (63.2)	
Partial or radical cystectomy	37 (24)	30 (81.1)	7 (18.9)	0.002*	14 (37.8)	23 (62.2)	0.152	29 (78.4)	8 (21.6)	<0.001*
Death										
No	138 (90)	85 (61.6)	53 (38.4)		40 (29.0)	98 (71.0)		62 (44.9)	76 (55.1)	
Yes	16 (10)	6 (37.5)	10 (62.5)	0.064	4 (25.0)	12 (75.0)	0.738	10 (62.5)	6 (37.5)	0.182

LN, lymph node; TURB, transurethral resection of the bladder.
Data are presented as n (%).
*p<0.05 was considered statistically significant, †Kruskal-Wallis test. All others were assessed using a χ^2 test.

cancer cells increased significantly among T24 (202%, 169%, and 224%, respectively) and 5637 (231%, 193%, and 206%, respectively) cells in comparison with colony numbers of control cells at 10 days (p<0.05) (Fig. 6A). Cell motility with wound generation indicated that cell migration in transfected cells was increased in comparison with control cells. After 24 h, we observed that EphB (B2, B3, and B4) transfected cells led to increased migrating cell numbers among T24 (280%, 190%, and 266%, respectively) and 5637 (457%, 325%, and 439%, respectively) cells, compared with control cells (p<0.05) (Fig. 6B). Matrigel invasion assay was applied to evaluate invasion activity of the tumor cells. Fig. 6C shows the staining results at 48 h. EphB (B2, B3, and B4) siRNA transfected cells increased significantly among T24 (317%, 315%, and 325%, respectively) and 5637 (261%, 241%, and 237%, respectively) cells in comparison with control cells (p<0.05). The EphB (B2, B3, and B4) transfected cells become more invasive cancer cells.

DISCUSSION

Clinicians predict the probabilities of bladder cancer progression and recurrence patients using several parameters, such as tumor grade, tumor invasion, and clinical staging. Of these,

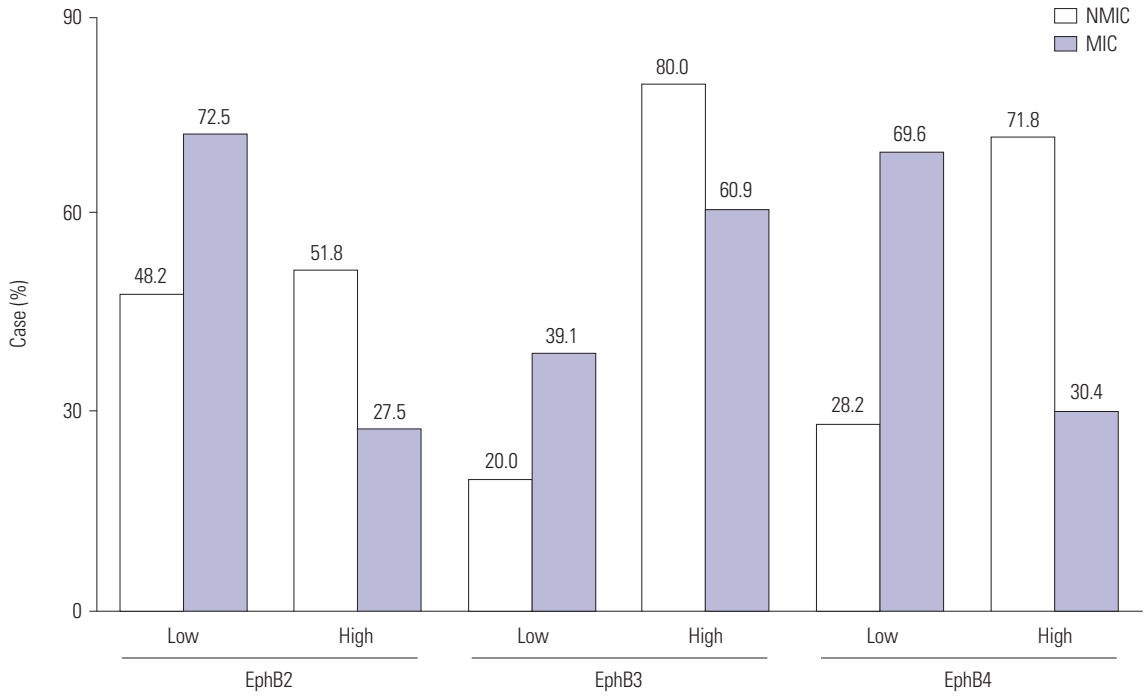

Fig. 2. Differences in EphB (B2, B3, and B4) expression according to muscular invasiveness of bladder cancer. NMIC, non-muscular invasive cancer; MIC, muscular invasive cancer.

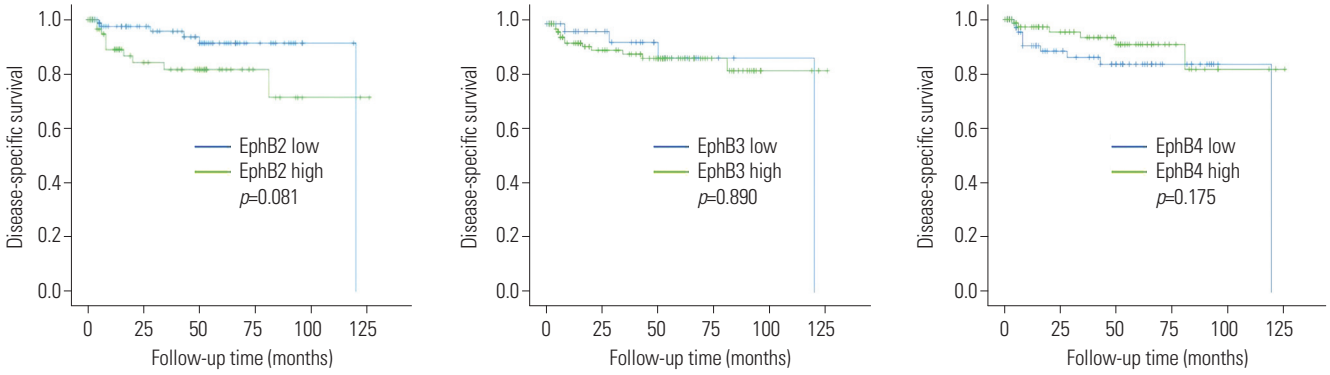

Fig. 3. Kaplan-Meier analyses of bladder cancer patient survival with respect to EphB (EphB2, B3, and B4) expression.

the most important factor is tumor invasiveness (T category). The recurrence rate of non-muscle-invasive bladder cancer is approximately 50–70%. About 2% of low-grade tumors and up to 45% of high-grade tumors progress to muscle-invasive cancer,[21] which has a 5-year survival rate of less than 50%.

Ephs/ephrins signaling has drawn growing interest in regards to their function; however, their biological significance is not totally understood. Eph receptor tyrosine kinase signaling affects both contacting cells and can modulate various biological responses.[22] Eph receptors guide invasion, adhesion, and epithelial phenotype by adjusting organization of the actin cytoskeleton, consequently directing the movement of tumor cells through their microenvironment.[23] Dysregulating mutations in Eph receptors has been found to play a part in cancer pathogenesis.[5] Even though bidirectional signaling promotes angiogenesis within tumor, its intrinsic role in tumor progression is complex and intriguing, as demonstrated by paradoxical effects. Eph protein expression has both tumor-promoting and tumor-suppressing effects in cancer. According to previous studies, Ephs/ephrins are often up-regulated in various malignant tumors and are correlated with high vascularity and poor prognosis in cancer, indicating their critical function in tumor progression.[24] Overexpression of Eph receptors, including EphA1, EphA3, EphB2, and EphB4, has been reported during carcinogenesis.[25] Meanwhile, however, both overexpression, and low expression of Eph receptor have been shown to be correlated with tumor progression. As well, some studies have indicated that overexpression of Eph receptors is associated with less malignant stage, while the loss of the Eph receptor is associated with more advanced stages.[5]

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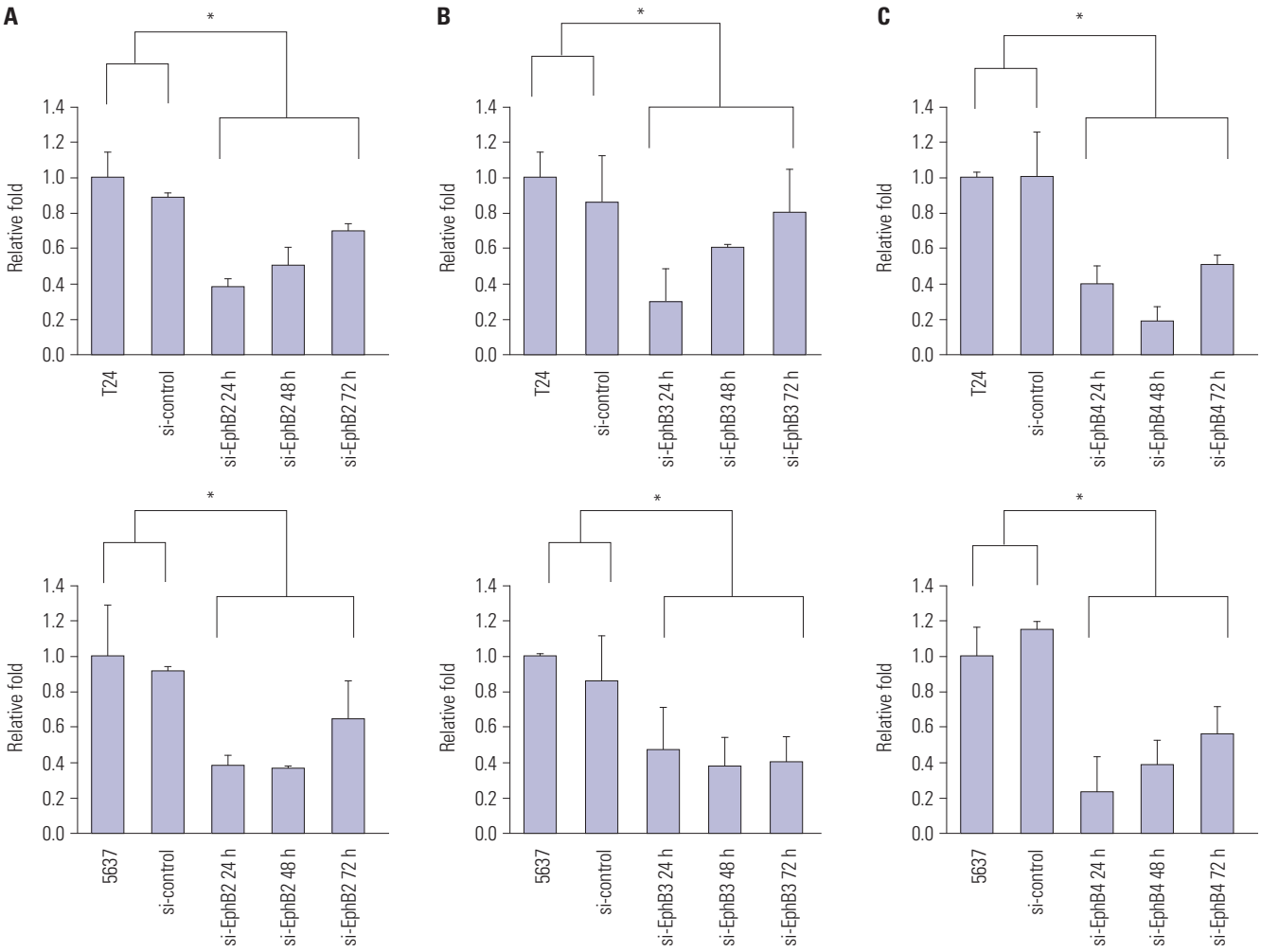

Fig. 4. EphB (B2, B3, and B4) expression by real-time PCR (RT-PCR) after siRNA transfection. RT-PCR analysis demonstrated that EphB siRNA suppressed EphB expression, compared with control cancer cells. A: EphB2. B: EphB3. C: EphB4. *p<0.05. siRNA, small interfering RNA.

A few studies have reported that EphB2 is a significant tumor suppressor gene in various tumor types. Batlle, et al.[18] reported that in colon cancer cells silencing of EphB2 lead to an invasive phenotype. Guo, et al.[15] described that EphB2 expression was progressively lost in each crucial point of colon cancer progression and that loss of EphB2 expression was associated with more advanced tumor stage and poor overall survival and liver metastasis.[26] In gastric cancer, loss of EphB2 expression is lost in more advanced stages and metastasis.[27] Recently, Li, et al.[16] described that EphB2 is largely expressed in normal bladder tissue, but lost in bladder cancer, possibly acting as a cell survival factor. Similarly, in our study of 154 cases, 91 (59%) cases demonstrated low expression of EphB2, which was significantly associated with higher tumor grade, muscular invasion, advanced stage, and a high incidence of cystectomy. Our in vitro studies showed that EphB2 inactivation increased bladder cancer cell proliferation, motility and invasion, implying that the loss of EphB2 contributes to tumor invasion and metastasis of bladder cancer.

EphB3 was first researched in the developing nervous system.[28] Administration of soluble ephrinB1 interfered with EphB3 function and elicited disorganization and a loss of migratory patterns of neural crest cell movement. In colorectal cancer, loss of EphB3 expression was found to result in aggressive adenocarcinoma in Apc (Min/+) mice, and EphB3 appeared to play a role as an inhibitor of tumorigenesis by regulating intercellular repulsive and adhesive interactions.[18] Moreover, the up-regulation of EphB3 expression was shown to increase cell-cell contact and to inhibit tumor growth in colon cancer.[29] Recently, Gao, et al.[30] reported that EphB3 expression was negatively associated with International Federation of Gynecologists and Obstetricians stage and histological grade in ovarian serous carcinomas. However, no study has investigated the role of EphB3 in bladder cancer. As far as we know, this is the first study to demonstrate the role of EphB3 expression in bladder cancer and we discovered that low expression of EphB3 was significantly associated with higher tumor grade and muscular invasion.

According to previous studies, the role of EphB4 is also controversial. Some studies have reported that EphB4 is up-regulated in bladder cancer, prostate cancer, and ovarian cancer.[9,11,12]

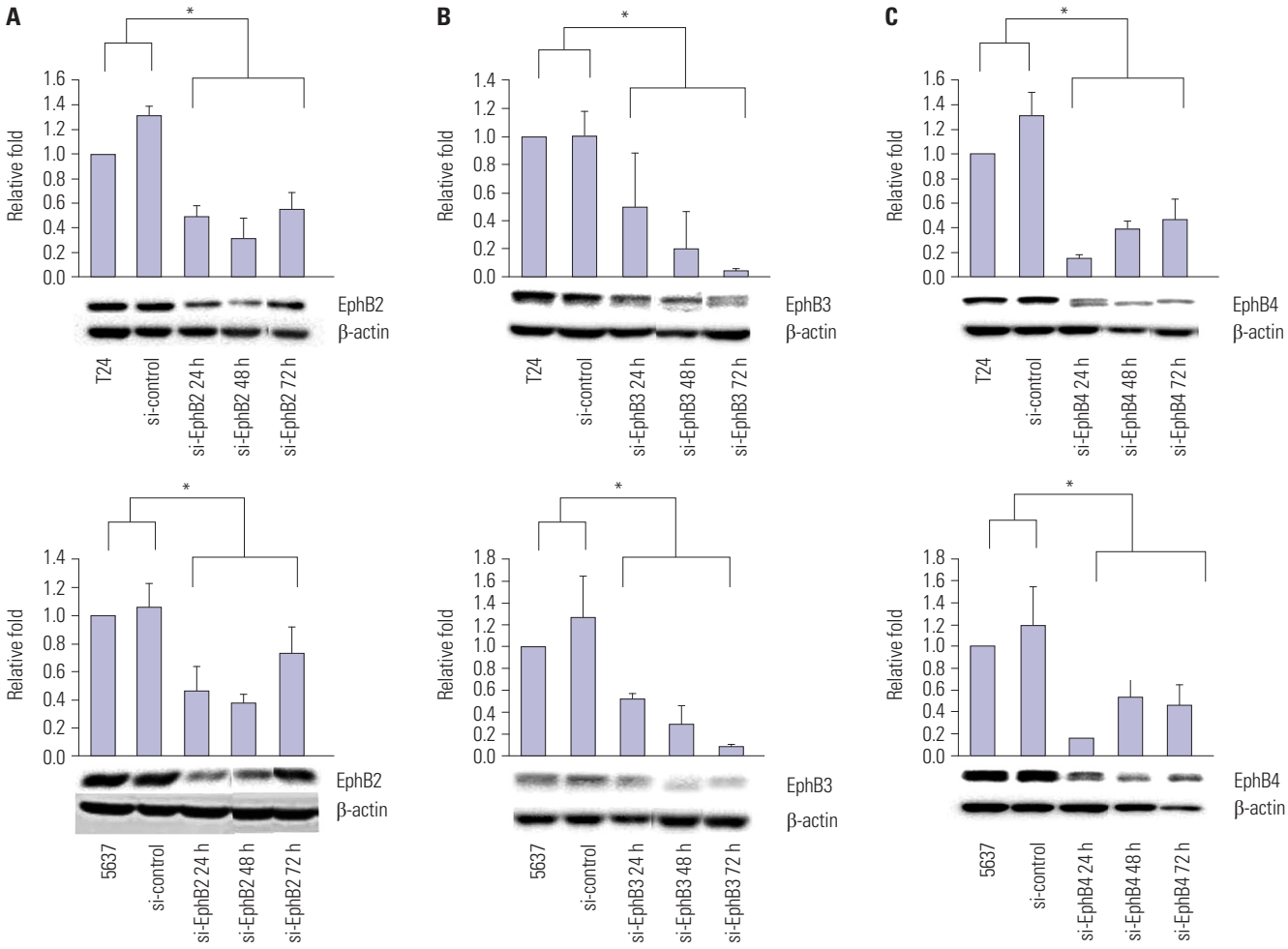

Fig. 5. EphB (B2, B3, and B4) expression by Western blot after siRNA transfection. Western blot analysis demonstrated that EphB expression was suppressed in the cancer cells transfected with EphB siRNA, compared with control cancer cells. β-actin was detected as a loading control. A: EphB2. B: EphB3. C: EphB4. *p<0.05. siRNA, small interfering RNA.

Nevertheless, other studies have shown that EphB4 suppresses tumor growth. In breast cancer, knockdown of EphB4 suppressed breast cancer cell invasion, viability, and migration in vitro and tumor growth in vivo.[10] The functional activation of EphB4 appears to play a role in tumor progression by enhancing angiogenesis via ephrinB2 in breast cancer.[31] Hu, et al.[32] reported that the status of EphB4 phosphorylation switched EphB4 from a tumor promoter to a tumor suppressor.

In this study, similar to the EphB2 and B3 results, EphB4 expression was reduced in 82 (53.2%) of the 154 cases, and this was correlated with tumor grade, muscular invasiveness, and advanced tumor stage. Contrary to our study, Li, et al.[16] reported that EphB4 is highly expressed in bladder cancer. However, the discrepancy may be in part explained by the relatively small number of cases investigated in their study (30 cases of invasive disease and four cases of superficial disease). Rutkowski, et al.[33] also reported that overexpression of EphB4 leads to increased migration, anchorage-independent growth, and invasion, which are correlated with an aggressive phenotype. That means the overexpression of EphB4 possibly promotes tumor progression. However, this result was reversed together with ephrinB2, which reduced EphB4 protein levels, showing that ligand-dependent signaling suppressed tumor growth in prostate cancer. In normal tissue of the breast, EphB4 expression was noted only in parenchymal cells. Remarkably, the number of cells with EphB4 expression has been found to be reduced in most invasive cancers analyzed. This suggests that EphB4 could be an effective agent for therapeutic intervention.[25] For therapeutic usage, EphB4 knockdown with antisense oligodeoxynucleotides has been found to increase apoptosis and to decrease breast cancer cell survival.[10]

To further define the functions of EphB2, B3, and B4 in bladder cancer invasion, we employed siRNA to knockdown the expression of EphB2, B3, and B4 in bladder cancer cells (T24, 5674). In doing so, we confirm that EphB2, B3, and B4 knockdown leads to increased migration, invasion, and proliferation of bladder cancer cells. This suggests that the activation of EphB2, B3, and B4 may be helpful for bladder cancer treatment.

Recently, three-dimensional organoid cultures have demonstrated the importance of the WNT/β-catenin pathway in blad-

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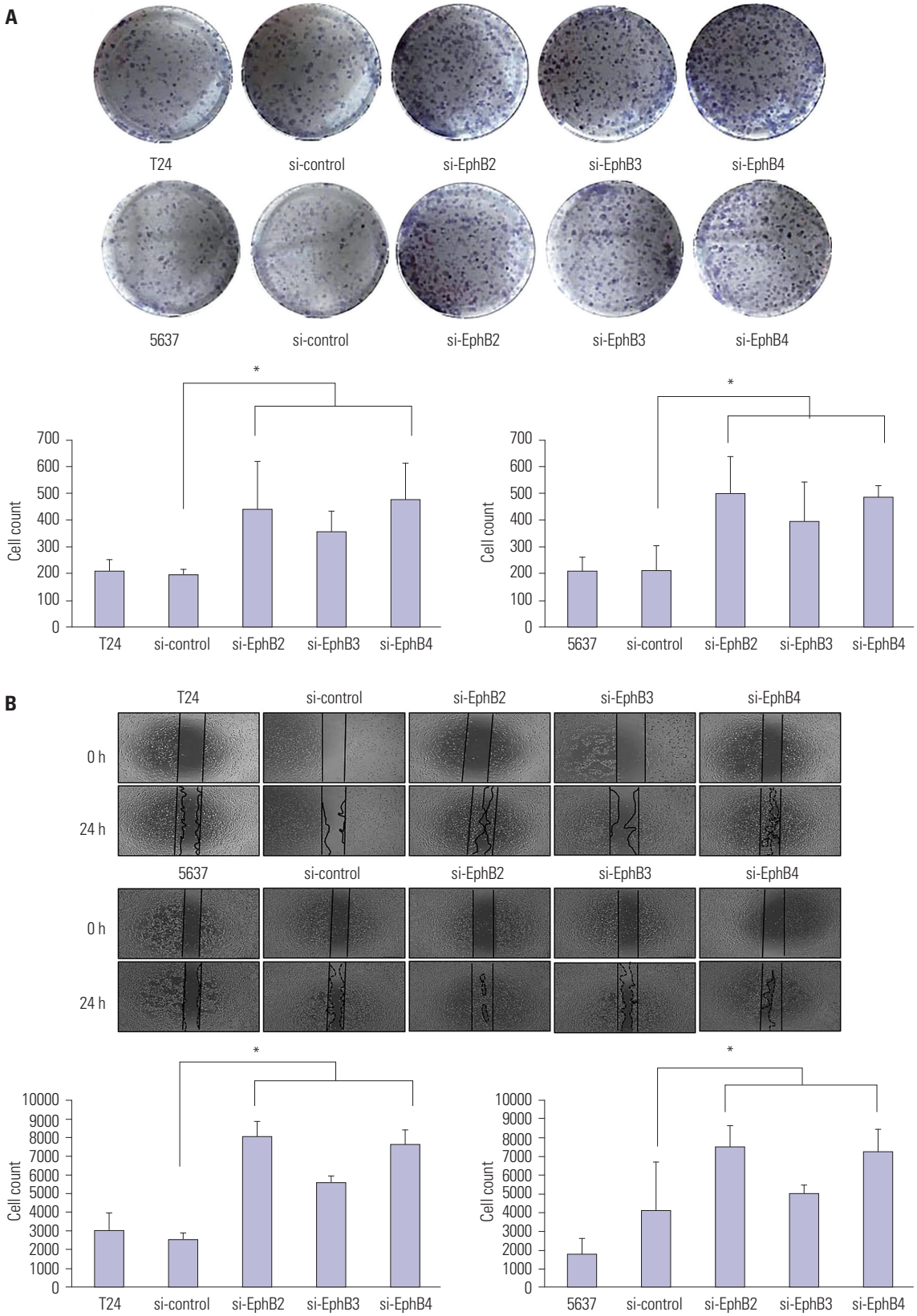

Fig. 6. Inhibition of EphB increases proliferation and invasion of bladder cancer cells (T24 and 5637). (A) Colony forming assay was performed to assess cell proliferation. The proliferation rate of the transfected cells was significantly faster than that of the control cells at 10 days. Colony numbers of transfected cells increased significantly, compared with that of control cells (10 days) (*p<0.05). (B) Cell migration (×100) was analyzed using wound-healing assay. Cell migration was increased significantly after EphB siRNA transfection, compared with that for control cells, after 24 h (*p<0.05). Cell invasion was significantly increased after EphB siRNA transfection, compared to control cells (*p<0.05). siRNA, small interfering RNA.

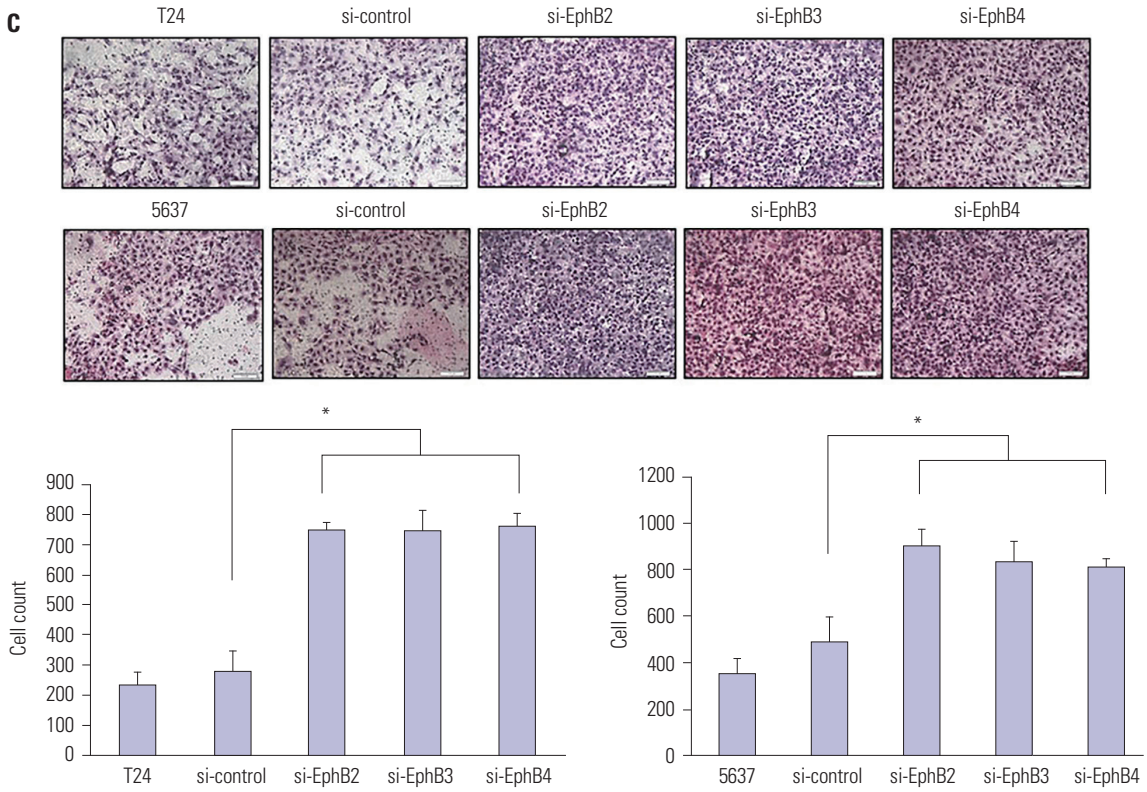

Fig. 6. Inhibition of EphB increases proliferation and invasion of bladder cancer cells (T24 and 5637). (C) Matrigel invasion assay was used to analyze cell invasion (×100). Cell invasion was significantly increased after EphB siRNA transfection, compared to control cells (*p<0.05). siRNA, small interfering RNA.

der cancer cell proliferation.[34] This study verified previous results indicating that the expression of activated β-catenin with PTEN deletion promote bladder cancer development.[35] EphB2 and EphB3 are β-catenin and T cell factor (Tcf) target genes in colorectal cancer. Low grade tumor areas exhibit an abundance of EphB positive cells, whereas clusters of EphB negative cells were equivalent to high grade areas in colorectal cancer. In colorectal cancer, EphB levels were found to be down-regulated despite Wnt signaling pathway activation, which suggested that cancer cells transcriptionally silenced EphB expression via tumor cell compartmentalization.[36] Similarly, in our study, EphB2, EphB3, and EphB4 were down-regulated in higher tumor grade and more advanced stages of bladder cancer. Meanwhile, androgen can promote bladder cancer progression and metastasis through Slug mediated epithelial-mesenchymal transition (EMT), due to WNT/β-catenin signaling pathway activation.[37] Mesenchymal-to-epithelial transition (MET) is the reverse process of EMT. EphB3 has been found to promote MET by enhancing cell-cell contact, and to suppress tumor growth in colorectal cancer.[29] Inhibition of EphB4 has been shown to lead to the disruption of cell junctions that favor EMT in breast cancer.[38] Thus, further investigation of EphB receptors and WNT/β-catenin signaling pathway in bladder cancer will be required.

There are some limitations to this study. Because non-muscle-invasive bladder cancer patients are initially treated with TURB and many of them have repeated surgery, specimen selection is difficult. In our study, specimens were obtained from TURB, partial cystectomy, or radical cystectomy cases; however, from a total of 154 specimens, only 20 specimens were obtained from repeated surgeries in 18 patients. Thus, our results are thought to be meaningful in that there was a large number of cases and a relatively small number of duplicate cases. EphB receptors bind 3 ephrinB ligands, with some exceptions: EphB2 binds ephrinA4, while EphB4 is known to bind ephrinB2 only.[5] EphB signaling is activated by receptors binding to their ligands, which results in the activation of downstream signaling; however, EphB itself can result in ligand-independent activation of downstream signaling.[22] The accurate function of EphB receptors in bladder cancer progression is not obvious. Further understanding about the signaling mechanisms of EphB2, B3, and B4 in bladder cancer cells will be required to devise treatment strategies targeting them.

In conclusion, observed correlations for low expression of EphB receptors (B2, B3, and B4) with muscular invasion indicated that EphB receptors may be key regulators of aggressive behavior and tumor progression in bladder cancer and potentially predictive markers of muscular invasion in bladder cancer patients.

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AUTHOR CONTRIBUTIONS

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