Steroid sulfatase promotes invasion through epithelial-mesenchymal transition and predicts the progression of bladder cancer

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Abstract. Androgen signal has been recently suggested to be associated with the progression of bladder cancer. Steroid sulfatase (STS) is a steroid sulfate activation enzyme, considered to be one of the key enzymes in the androgen signaling pathway. However, the role of STS in bladder cancer has not been elucidated. The purpose of the present study was to determine the clinical and functional significance of STS in bladder cancer. Immunohistochemical analysis of surgical specimens obtained by radical cystectomy (n=114) demonstrated that overexpression of STS was associated with the invasion of bladder cancer, as evidenced by the incidence of STS-positive cancers (11.5 and 37.1% in non-muscle invasive and muscle invasive bladder cancers, respectively; P=0.003). STS-positive cancer demonstrated shorter recurrence-free survival and cancer-specific survival (P=0.0027 and 0.0030, respectively). Furthermore, knockdown of STS significantly reduced cell migration and invasion capacities of bladder cancer cells (P<0.001 and P=0.005, respectively), accompanied by the upregulation of E-cadherin and downregulation of vimentin. In summary, the present study demonstrated that STS promotes the invasion capability of bladder cancer via regulation of the epithelial-mesenchymal transition, and may be a useful marker for predicting the progression of bladder cancers.

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Abbreviations: STS, steroid sulfatase; RFS, recurrence-free survival; CSS, cancer-specific survival; AR, androgen receptor; DHT, dihydrotestosterone; EMEM, Eagle's minimum essential medium; MIBC, muscle-invasive bladder cancer; NMIBC, non-muscle-invasive bladder cancer

Key words: steroid sulfatase, recurrence-free survival, cancer-specific survival, bladder cancer, epithelial-mesenchymal transition

Introduction

Males have a higher incidence of bladder cancer than females (1). Although cigarette smoking and occupational exposure to chemicals are the common risk factors associated with the development of bladder cancer, the higher incidence in males cannot be fully explained even after adjustment for these carcinogenic factors (2-4). Recent findings have suggested that androgen signals could account, in part, for the gender difference in bladder cancer incidence (5-7). Androgen receptor (AR) and dihydrotestosterone (DHT) are the most important factors in androgen signals. Although *in vitro* and animal studies have demonstrated that AR serves an important role in bladder cancer (6,7), no significant association was observed between AR expression and pathological stage, grade or outcome of bladder cancer in a recent multi-institute study (8). DHT has also been demonstrated to promote the growth of bladder cancer cells both *in vitro* and in animals (6,9), but there is no report on the expression of enzymes involving the formation of DHT in patients with bladder cancer.

Dehydroepiandrosterone is one of the sources of DHT, which is converted from dehydroepiandrosterone sulfate by steroid sulfatase (STS) (10). Dehydroepiandrosterone, having higher circulating concentration and longer half-life in blood, acts as a central reservoir for the formation of biologically active androgens via the action of tissue STS (10). Inhibition of STS may be a novel approach to block the formation of steroids with potent androgenic property; STS has also been suggested to be a therapeutic target for androgen-dependent cancer, such as prostate cancer (11,12), in which STS expression was observed in 85% of cancer tissues, but not in normal tissues (13). To the best of our knowledge the expression of STS in bladder cancer has not yet been studied. The purpose of the present study was to determine the clinical and functional signification of STS in bladder cancer.

Materials and methods

Patients. Immunohistochemical analysis was performed on samples from 114 patients with urothelial carcinoma, who were treated for bladder cancer by radical cystectomy at Osaka City University Hospital between January 1995 to December 2015.

The clinicopathological characteristics of the patients are summarized in Table I. There were 89 male and 25 female patients, and the median age was 69 years (range, 33-84 years). Patients who were either incompletely resected, or histologically confirmed as small cell carcinoma of the bladder, or lost to follow-up, were excluded from the study. Pathologic staging was performed according to the 2009 Tumor, Node, Metastasis classification system (14), and grading was done according to the criteria by the World Health Organization, 2004 (15). The Institutional Review Board at Osaka City University Graduate School of Medicine approved the use of the specimens and clinical data in accordance with the Declaration of Helsinki and guidelines of Osaka City University Graduate School of Medicine (study approval no. 1955). All 114 patients included in the present study provided written informed consent for the collection and use of tissue samples and for the publication of their results.

Immunohistochemical analysis of STS, E-cadherin and vimentin in bladder cancer tissues. Tissues obtained by radical cystectomy were fixed with 10% formalin for 24-48 h at room temperature and paraffin-embedded. Paraffin embedding was performed as follow: 100% ethanol (4x1.5 h at 37˚C), 100% ethanol (2x2 h at 37˚C), 100% ethanol (3 h at 37˚C), 100% xylene (3x30 min at 35˚C), paraffin wax (2x30 min at 58˚C), paraffin wax (30 min at 59˚C), paraffin wax (30 min at 60˚C) and embedding of the tissues into paraffin blocks. Tissues were cut into 3-μm sections for histological analysis. Tissues were stained with Mayer's hematoxylin solution for 5 min and counterstained in eosin Y ethanol solution for 3-5 min both at room temperature. Formalin-fixed, paraffin-embedded tissues of bladder cancer were analyzed by immunohistochemical staining as described previously (4,16). Sections were incubated with rabbit polyclonal antibody to STS (HPA 002904; 1:100; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), rabbit polyclonal antibody to E-cadherin (sc-7870; 1:100; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and rabbit monoclonal antibody to vimentin (D21H3; 1:100; Cell Signaling Technology, Inc., Danvers, MA, USA) at 4˚C overnight. This was followed by incubation with biotinylated goat anti-rabbit IgG (BA-4000; 1:200; Vector Laboratories, Inc., Burlingame, CA, USA) for 30 min at room temperature. Immunoreactivity was detected using a VECSTAIN Elite ABC kit (PK-6101; Vector Laboratories, Inc.) and 3,3'-diaminobenzidine hydrochloride (Sigma-Aldrich, St Louis, MO, USA).

Immunohistochemical analysis was performed by three pathologists. Immunoreactivity of STS was observed in the cytoplasm of bladder cancer cells, but not in normal urothelium. A benign prostate tissue, which was simultaneously removed at the time of radical cystectomy, was used as a positive control. Tissues with >5% cancer cells immunoreactive for STS were defined as positive (17). Evaluation of staining for E-cadherin and vimentin were performed based on a staining index (18,19).

Cell lines. The human bladder cancer cell lines (5637, HT1376, UMUC3, TCCSUP and T24) and prostate cancer cell lines (LNCaP and PC-3) were purchased from the American Tissue Culture Collection (Manassas, VA, USA). Cells were authenticated by short tandem repeat analysis performed by Takara Bio, Inc. (Otsu, Japan) and tested to ensure that they were mycoplasma-free by DDC Medical (Thermo Fisher Scientific, Inc., Waltham, MA, USA) in November 2017. Cells were maintained as monolayer cultures at 37˚C and 5% CO_2. The 5637 cell line was grown in RPMI-1640 (Sigma-Aldrich; Merck KGaA) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich; Merck KGaA), 1% HEPES, and 1% D-Glucose. HT-1376 was grown in Eagle's minimal essential medium (EMEM; Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% FBS, 1% sodium pyruvate solution (Sigma-Aldrich; Merck KGaA) and 1% MEM non-essential amino acid solution (Thermo Fisher Scientific, Inc.). UMUC3 and TCCSUP were grown in EMEM, LNCaP and PC-3 in RPMI 1640, and T24 in McCoy's 5A medium (Thermo Fisher Scientific, Inc.), supplemented with 10% FBS.

Table I. Patients' characteristics and steroid sulfatase expression (n=114).

Characteristic	Steroid sulfatase-positive tumors, n (%)	P-value
Age, years		0.758
<65 (n=30)	7 (23.3)	
≥65 (n=84)	22 (26.2)	
Sex		0.852
Male (n=89)	23 (25.8)	
Female (n=25)	6 (24.0)	
Pathological Stage		0.003
pTa + pT1 + pTis (n=52)	6 (11.5)	
pT2 + pT3 + pT4 (n=62)	23 (37.1)	
Grade		0.561
Low (n=11)	2 (18.2)	
High (n=103)	27 (26.2)	
Lymph node involvement		0.065
Yes (n=13)	6 (46.2)	
No (n=101)	23 (22.6)	
Neoadjuvant chemotherapy		0.844
Yes (n=45)	11 (24.4)	
No (n=69)	18 (26.1)	
Smoking history		0.648
Yes (n=47)	13 (27.7)	
No (n=67)	16 (23.9)	

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cell lines using RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. RT-qPCR assay was performed as described previously (4). The catalogue numbers for the primers used for qPCR were as follows: STS, Hs00996676_m1; E-cadherin, Hs01023894_m1; Vimentin, Hs00185584_m1; GAPDH, Hs00266705_g1 (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were: 20 sec at 95˚C followed by 40 cycles of 3 sec at 95˚C and 30 sec at 60˚C. Data were then quantified using

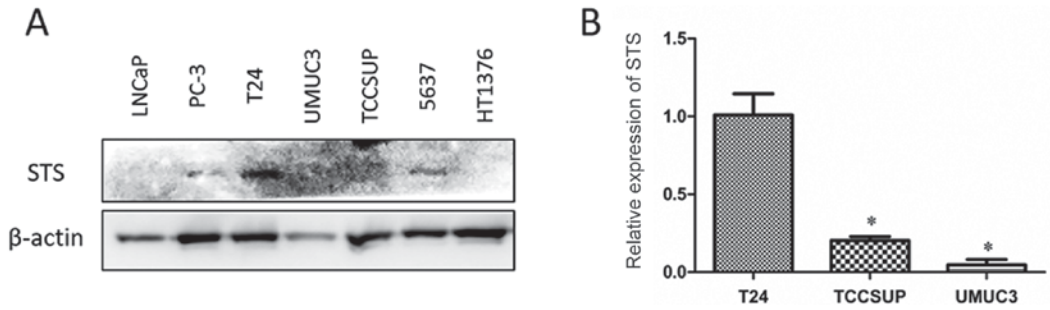

Figure 1. Expression of STS in prostate cancer and bladder cancer cell lines. (A) Protein expression levels of STS are presented in prostate cancer and bladder cancer cell lines. (B) mRNA expression levels of STS are presented in T24, TCCSUP, and UMUC3 cells. Relative STS expression levels have been normalized to those in T24 cells. Data are presented as the mean ± standard deviation. *P<0.01 vs. T24 cells. STS, steroid sulfatase.

the $2^{-\Delta\Delta Cq}$ method for relative gene expression (20), compared to that of GAPDH as internal control.

Western blot analysis. Western blotting was performed as previously described (4). Primary antibodies for STS (1:1,000) and β-actin (ab8226; 1:1,000; Abcam, Cambridge, UK) were used for the present study. Goat anti-rabbit horseradish peroxidase (HRP)-conjugated IgG and goat anti-mouse HRP-conjugated IgG (nos. sc-2004 and sc-2005; 1:10,000; Santa Cruz Biotechnology) were used as secondary antibodies. Immunoreactive bands were visualized using ECL Prime Western Blotting Detection reagent (GE Healthcare, Chicago, IL, USA).

Knockdown of STS. STS expression was transiently knocked down in T24 cells using Lipofectamine™ RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. STS-specific small interfering (si)RNAs (Silencer® Select siRNAs) were obtained from Thermo Fisher Scientific, Inc. The sense sequences of siRNA for STS were as follows: si-STSA, 5'-CUAGCAACAUGG ACAUAUUTT-3'; and si-STS B, 5'-GGACAUAUUUCCUAC AGUATT-3'. Non-targeting control siRNA (cat. no. 4390844; Silencer® Select Negative Control siRNA) was obtained from Thermo Fisher Scientific, Inc. T24 cells ($1.5x10^5$) were transiently transfected with 10 nM si-STS A, si-STS B, or control siRNA in a 6-well plate. Following 48 h, cells were trypsinized and used in additional assays.

Cell viability assay. To investigate the effect of STS knockdown on cell proliferation, transfectants ($1x10^4$/well) were seeded in a 96-well plate and grown in McCoy's 5A medium supplemented with 10% FBS at 37˚C with 5% CO_2. After 48 h, cell proliferation was measured using a Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's instructions. The number of cells was measured using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 450 nm.

Cell migration and invasion assay. Migration assay was performed using a Cell Culture Insert with an 8.0-μm pore size PET filter (BD Biosciences, Franklin Lakes, NJ, USA) and invasion was assessed via a Matrigel invasion assay (BD Biosciences), according to the manufacturer's protocol. Briefly, 2-3x10^4 cells in 500 μl serum-free McCoy's 5A medium were seeded in the upper chamber, whereas the lower chamber was loaded with medium containing 10% FBS. Following a 24-h incubation at 37˚C, the cells that remained inside the inserts were removed with cotton swabs, and those that migrated or invaded the reverse side of the inserts were fixed with 5% glutaraldehyde for 15 min and stained with Giemsa for 30 min both at room temperature. The cells that had migrated or invaded through the membranes were counted by light microscopy (magnification, x20).

Statistical analysis. Statistical analyses were performed with GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA). Fisher's exact test was used to evaluate the differences in incidence of STS expression patterns among clinical and pathological parameters. The recurrence-free survival was defined as the time between the date of surgery and the last date of follow-up or the date of recurrence. The curves were analyzed using the Kaplan-Meier method with the log-rank test to assess statistical significance. For multiple analyses, Cox proportional hazards analysis was used to determine the relative contribution of various factors to the risk of progression. One-way ANOVA followed by a Sidak's post hoc test was used to assess the difference between the *in vitro* assays. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of STS, E-cadherin and vimentin in cell lines and bladder cancer samples. Western blot analysis demonstrated a strong expression of STS in T24 cells, and a weak expression in PC-3 and 5637 cells, compared with those in other cell lines (Fig. 1A). The mRNA expression levels of STS were analyzed by RT-qPCR in three bladder cancer cell lines with invasive characteristics, T24, TCCSUP and UMUC3 cells (Fig. 1B). The expression level of STS was significantly higher in T24 cells than in TCCSUP and UMUC3 cells; therefore, T24 was selected for an *in vitro* knockdown assay.

Immunohistochemical analysis of STS was performed using 114 formalin-fixed, paraffin-embedded tissues from patients with bladder cancers (Fig. 2). Normal urothelium (Fig. 2A and D), non-invasive bladder cancer (Fig. 2B and E) and invasive bladder cancer (Fig. 2C and F) were compared with benign prostate tissue (Fig. 2G) as a positive control of STS. Immunoreactivity of STS was observed in the cytoplasm of bladder cancer cells in invasive areas of cancer (Fig. 2E), but not

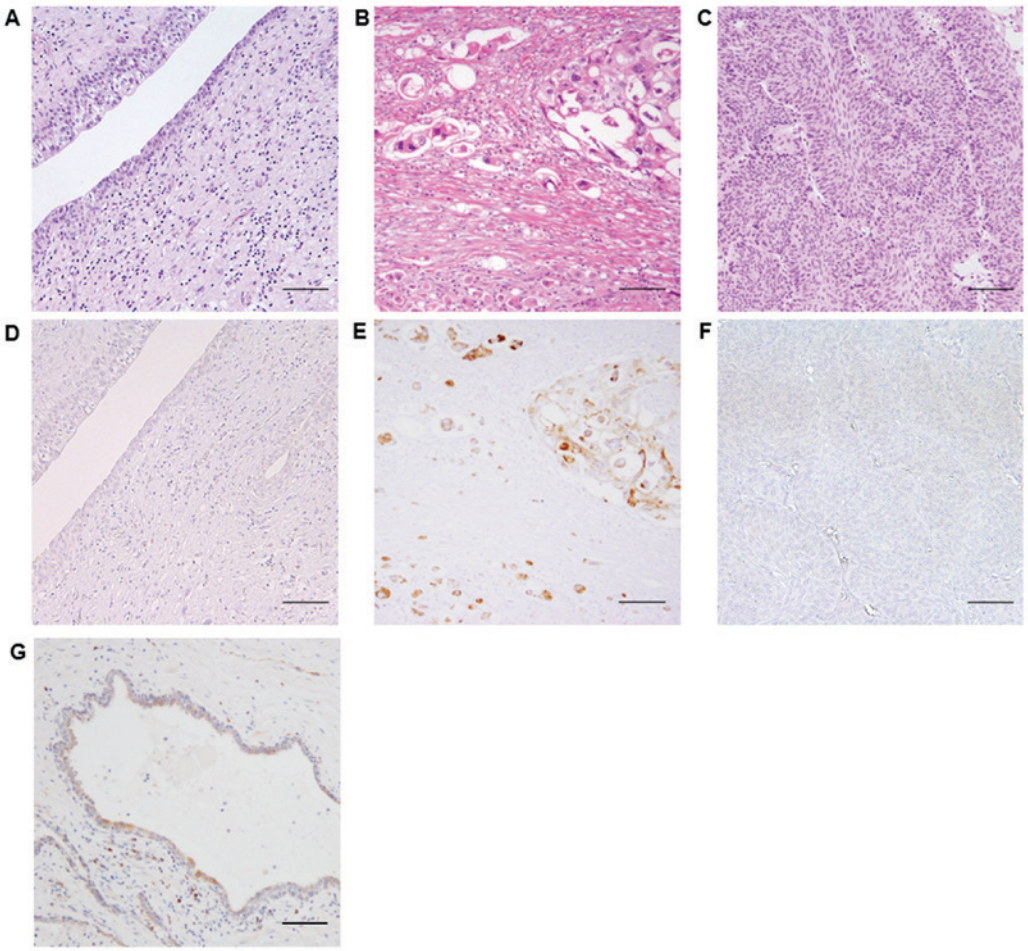

Figure 2. (A-C) Hematoxylin and eosin staining and (D-G) immunohistochemical staining of STS in bladder cancer tissues. STS was not expressed in either (A and D) normal urothelium or (C and F) non-muscle invasive bladder cancer tissues, but was highly expressed in (B and E) the cytoplasm of invasive bladder cancer cells. (G) Benign prostate tissue was used as a positive control. Scale bar indicates 100 μm. STS, steroid sulfatase.

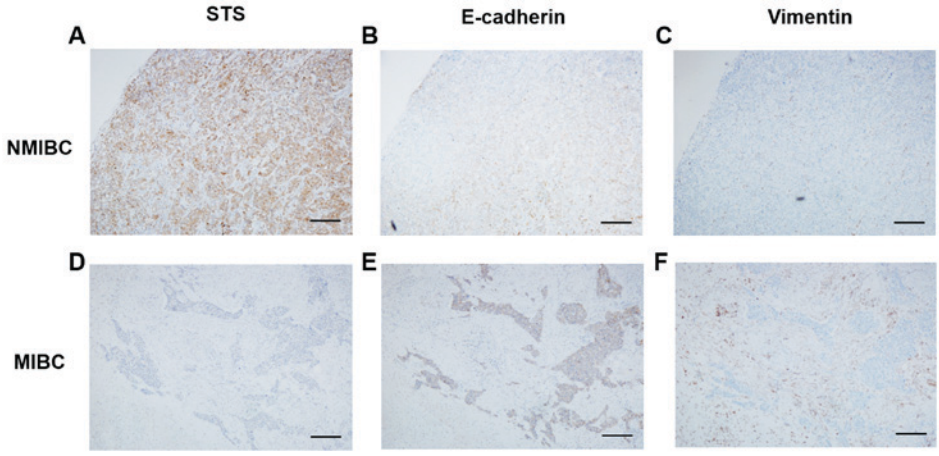

Figure 3. Expressions of STS, E-cadherin and vimentin in bladder cancer tissues. Representative staining is presented for (A-C) NMIBC and (D-F) MIBC. In an NMIBC tissue that was (A) positive for STS, (B) E-cadherin was positive and (C) vimentin was negative. In the other MIBC tissue that was (D) negative for STS, (E) E-cadherin was positive and (F) vimentin was negative. In many of the tissues, E-cadherin was positive and vimentin was negative regardless of the status of the positivity of STS and muscle-invasiveness. Scale bar indicates 200 μm. STS, steroid sulfatase; NMIBC, non-muscle-invasive bladder cancer; MIBC, muscle-invasive bladder cancer.

in the surface regions, especially in papillary tumors (Fig. 2F). As summarized in Table I, the incidence of STS-positive cancers was significantly higher in muscle invasive bladder cancers (MIBCs; pT2 + pT3 + pT4; 37.1%) than in non-muscle invasive bladder cancers (NMIBCs; pTa + pT1 + pTis; 11.5%). No significant association was demonstrated with age or sex. Immunohistochemical analyses of E-cadherin and vimentin were performed using 10 NMIBC and 10 MIBC tissues to

Table II. Association between steroid sulfatase and epithelial-mesenchymal transition-related factors by immunohistochemical analysis of E-cadherin and vimentin in non-muscle-invasive bladder cancer (n=10) and muscle-invasive bladder cancer (n=10).

A, Steroid sulfatase positive cancers

Parameter	Cancer incidence, n (%)	P-value
E-cadherin		0.531
Positive (n=17)	9 (52.9)	
Negative (n=3)	1 (33.3)	
Vimentin		0.224
Positive (n=2)	2 (100)	
Negative (n=18)	10 (55.6)	

B, Muscle invasive bladder cancers

Parameter	Cancer incidence, n (%)	P-value
E-cadherin		0.0603
Positive (n=17)	7 (41.2)	
Negative (n=3)	3 (100)	
Vimentin		0.136
Positive (n=2)	2 (100)	
Negative (n=18)	8 (44.4)	

NS, not significant.

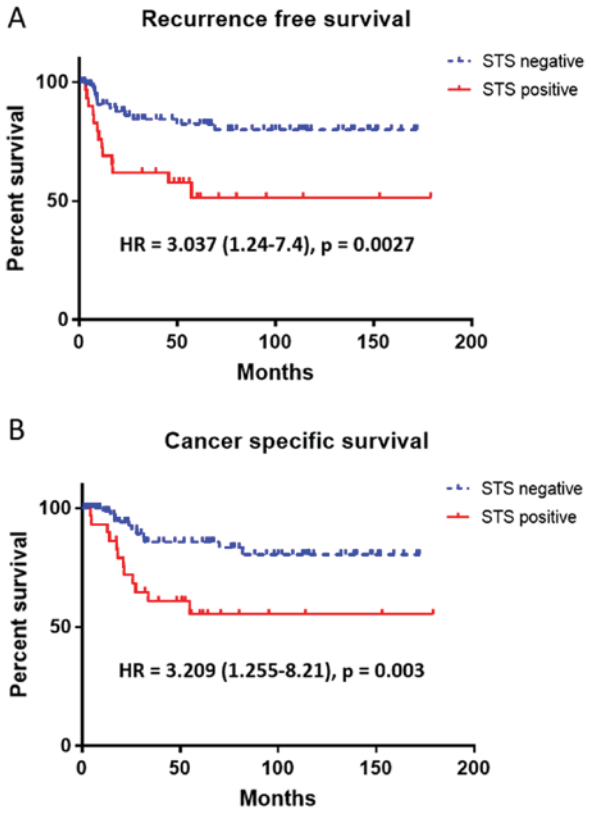

Figure 4. RFS and CSS in patients treated with radical cystectomy (n=114). STS-positive cancers exhibited shorter (A) RFS and (B) CSS. RFS; recurrence-free survival, CSS; cancer-specific survival; STS, steroid sulfatase.

analyze the association between STS and these EMT-related factors (Fig. 3). The expression of E-cadherin was positive in 85% (17/20) of tissues, while vimentin was negative in 90% (18/20). There was no statistical significance in the positivity of E-cadherin and vimentin between NMIBCs and MIBCs. Also, no significant association was observed in the expression between STS and these EMT-related markers (Table II).

Follow-up of outcome in patients and survival analysis. Correlation analysis of STS expression with clinical outcomes in 114 patients with bladder cancer revealed worse survival rates in patients with STS-positive cancer. Patients with STS-positive cancer exhibited shorter recurrence-free survival [RFS; Fig. 4A; hazard ratio (HR)=3.037; P=0.0027] and cancer-specific survival (CSS; Fig. 4B; HR=3.209; P=0.003) than those with STS-negative cancers. For patients treated with radical cystectomy, univariate analyses of clinicopathological parameters and RFS or CSS revealed the pathological stage (NMIBC vs. MIBC), lymph node metastasis and STS expression as the major risk factors (Table III). Multivariate analysis demonstrated that stage (MIBC vs. NMIBC) and positive lymph node involvement were independent risk factors for RFS, and stage (MIBC vs. NMIBC) was the only independent risk factor for CSS (Table IV).

Effect of STS knockdown on cell proliferation, migration, and invasion of bladder cancer cells. STS-specific siRNA reduced STS mRNA expression levels by 86-90% compared with that in the negative control cells (Fig. 5A). Knockdown of STS did not significantly affect cell proliferation (Fig. 5B), but significantly inhibited cell migration (35-80%; Fig. 5C) and invasion (66-73%; Fig. 5D).

Effect of STS knockdown on the expression of E-cadherin and vimentin. Total RNA was extracted from T24 cells transfected with negative control siRNA, si-STS A or si-STS B. RT-qPCR demonstrated that expression of E-cadherin was significantly upregulated (fold change, 6.75-6.77) and that of vimentin was significantly downregulated (fold change, 0.44-0.60) by STS knockdown (Fig. 5E and F).

Discussion

In the present study, it was demonstrated that the level of STS expression was significantly higher in MIBCs than in NMIBCs. It was also demonstrated that STS-positive bladder cancers exhibited shorter RFS and CSS. Furthermore, knockdown of STS inhibited cell migration and invasion of bladder cancer cells, via the regulation of E-cadherin and vimentin. To the best of our knowledge, the present study is the first to demonstrate the association between STS expression and invasion/progression of bladder cancers.

Epithelial-mesenchymal transition (EMT) is the critical process of invasion by which epithelial cells lose their intracellular adhesion and acquire a mesenchymal phenotype (21). Loss of E-cadherin is the most well known change during

Table III. Univariate analyses of clinicopathological parameters and the survivals of patients who were treated with radical cystectomy (n=114).

	Recurrence-free survival		Cancer-specific survival	
Variable	HR (95% CI)	P-value	HR (95% CI)	P-value
Stage	5.977 (2.769-12.900)	0.0002	7.25 (3.199-16.430)	0.0002
Grade	1.393 (0.396-4.907)	0.6504	1.44 (0.411-5.051)	0.6195
Lymph node involvement	5.117 (1.369-19.130)	<0.0001	5.439 (1.298-22.800)	<0.0001
STS	3.037 (1.247-7.400)	0.0027	3.209 (1.255-8.210)	0.0030
Neoadjuvant chemotherapy	2.837 (1.248-6.447)	0.0057	2.802 (1.381-5.683)	0.0015
Smoking history	1.067 (0.470-2.424)	0.8049	1.471 (0.750-2.886)	0.2433

HR, hazard ratio; CI, confidence interval; STS, steroid sulfatase.

Table IV. Multivariate analyses of clinicopathological parameters and the survival of patients who were treated with radical cystectomy (n=114).

	Recurrence-free survival		Cancer-specific survival	
Variable	HR (95% CI)	P-value	HR (95% CI)	P-value
Stage	4.686 (1.547-14.203)	0.0063	10.004 (1.259-79.474)	0.0294
Lymph node involvement	2.8716 (1.287-6.410)	0.01	2.023 (0.691-5.919)	0.1983
STS	1.2496 (0.857-1.823)	0.2472	1.4753 (0.895-2.436)	0.1285

HR, hazard ratio; CI, confidence interval; STS, steroid sulfatase.

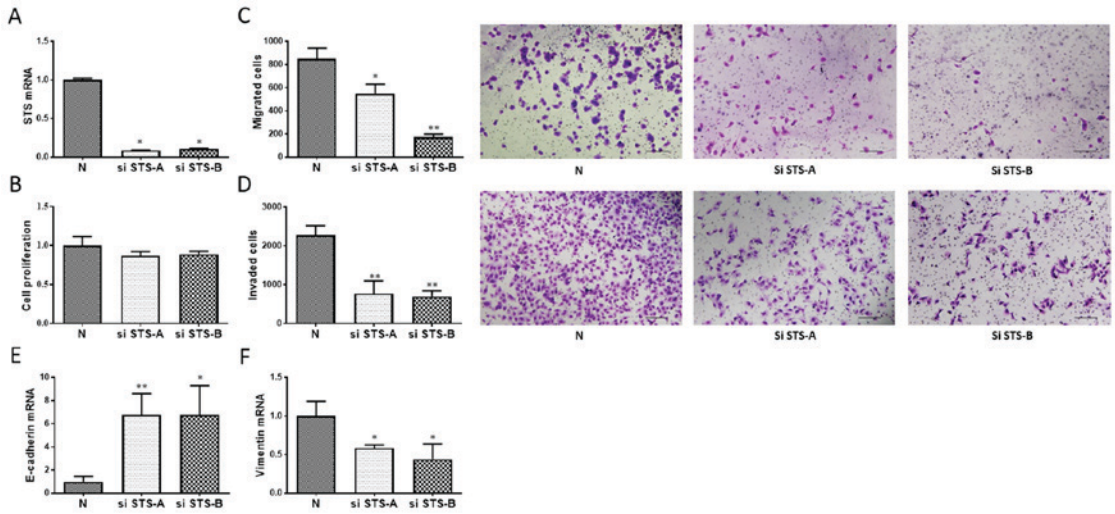

Figure 5. Effect of STS knockdown in T24 cells. (A) Two siRNAs for STS (si-STS A and si-STS B) reduced mRNA expression of STS in T24 cells. Relative STS expression levels are presented normalized to those in N cells. (B) Effect of siRNA knockdown on cell proliferation was evaluated using T24 cells. Each cell count was normalized to that of the N group. No significant difference was observed between si-STS-transfected and N cells. Effect of siRNA knockdown on cell migration and invasion ability was also evaluated using T24 cells. si-STS A- and B-transfected cells exhibited significantly reduced (C) migration and (D) invasion abilities compared with N cells. Scale bar indicates 500 μm. (E and F) Reverse transcription-quantitative polymerase chain reaction analyses of T24 cells transfected with siRNAs. Each gene expression level was normalized to that in N cells. (E) E-cadherin was upregulated and (F) vimentin was significantly downregulated by STS knockdown. *P<0.05 and **P<0.01 vs. negative control.. siRNA, small interfering RNA; STS, steroid sulfatase; si-STS, STS siRNA; N, negative control.

EMT. In contrast, mesenchymal markers such as vimentin, induce EMT (22). In a study by McConkey *et al*, a strong inverse correlation was observed between the expression of E-cadherin and vimentin in bladder cancer (23). The present

study demonstrated that knockdown of STS inhibited the invasion capacities of bladder cancer cells accompanied by the upregulation of E-cadherin and downregulation of vimentin, thereby suggesting that STS promoted invasion of bladder cancer by modifying EMT.

A major limitation of the present study, however, is the small sample size. STS could not be indicated as an independent prognostic factor for CSS of the patients with bladder cancer due to the small number of cancer-specific mortalities. Also, the association of STS and EMT-related markers could not be demonstrated by immunohistochemistry. Unexpectedly, E-cadherin was positive in 70% and vimentin was negative in 80% of MIBC tissues. Alteration of the expression levels of E-cadherin and vimentin might not occur simultaneously with the expression of STS in the tissue of bladder cancer cells. To clarify the significance of STS expression in clinical outcomes, further research is required. Another limitation of the present study was that it could not demonstrate whether the role of STS in bladder cancer invasion was dependent on androgen/DHT signaling. Accordingly, further study on the interaction network between STS and AR would elucidate the behavior of STS in EMT.

With respect to the mechanism underlying the upregulation of STS in cancers, Suh *et al* reported that phosphatidylinositol 3-kinase/protein kinase B activation mediates the induction of STS expression by tumor necrosis factor-α in human prostate and breast cancer cells (24). Hughes *et al* previously reported that stimulation of the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathways by 1α, 25-dihydroxyvitamin D3 may contribute to the increase in STS expression (25). Shin *et al* reported that STS induced Wnt/β-catenin signaling and EMT transition in human prostate and cervical cancer cells (26). Further studies to evaluate phosphatidylinositol 3-kinase/protein kinase B and ERK/MAPK pathways would facilitate a better understanding of the role of STS in invasive bladder cancer. In conclusion, the present study demonstrates that STS could promote the invasion capability of bladder cancer via regulating EMT, and could be a useful marker for predicting the progression of bladder cancers and a novel target for clinical therapy.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MK, HW, ST and MG were responsible for the conception and design of the present study; YS, MK, MF, AK and YT were responsible for the acquisition of data; and YS, MK, HW, TN and MG were responsible for the analysis and/or interpretation of data.

Ethics approval and consent to participate

The Institutional Review Board at Osaka City University Graduate School of Medicine (Osaka, Japan) approved the use of the specimens and clinical data in accordance with the Declaration of Helsinki and guidelines of Osaka City University Graduate School of Medicine (study approval no. 1955). All 114 patients provided written informed consent for the collection and use of their samples for the present study.

Patient consent for publication

All 114 patients included in this study provided written informed consent for the publication of their results.

Competing interests

The authors declare that they have no competing interests.

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