Mutation of TGF-β receptor II facilitates human bladder cancer progression through altered TGF-β1 signaling pathway

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Abstract. Tumor cells commonly adapt survival strategies by downregulation or mutational inactivation of TGF-β receptors thereby reversing TGF-β1-mediated growth arrest. However, TGF-β1-triggered signaling also has a protumor effect through promotion of tumor cell migration. The mechanism(s) through which malignant cells reconcile this conflict by avoiding growth arrest, but strengthening migration remains largely unclear. TGF-βRII was overexpressed in the bladder cancer cell line T24, concomitant with point mutations, especially the Glu^{269} to Lys mutation (G→A). Whilst leaving Smad2/3 binding unaffected, TGF-βRII mutations resulted in the unaffected tumor cell growth and also enhanced cell mobility by TGF-β1 engagement. Such phenomena are perhaps partially explained by the mutated TGF-βRII pathway deregulating the p15 and Cdc25A genes that are important to cell proliferation and CUTL1 gene relevant to motility. On the other hand, transfecting recombinant TGF-βRII-Fc vectors or smad2/3 siRNA blocked such abnormal gene expressions. Clinically, such G→A mutations were also found in 18 patients (n=46) with bladder cancer. Comparing the clinical and pathologic characteristics, the pathologic T category (χ^2 trend = 7.404, P<0.01) and tumor grade (χ^2 trend = 9.127, P<0.01) tended to increase in the G→A mutated group (TGF-βRII point-mutated group). These findings provide new insights into how TGF-β1 signaling is tailored during tumorigenesis and new information into the current TGF-β1-based therapeutic strategies, especially in bladder cancer patient treatment.

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Introduction

Bladder cancer is one of the most common urologic malignances with over 300,000 new cases worldwide diagnosed annually (1,2). Despite the advances of chemotherapeutics, no improvement in survival of advanced or metastatic bladder cancer has been reported (3-7). Intense studies on molecular mechanisms have led to the identification of numerous signaling pathways involving bladder carcinogenesis, which provide new hope for bladder cancer treatment. Among them, the following features of TGF-β1 signaling pathway has attracted much attention: i) the association of changes affecting either the level of TGF-β1 or the expression of its receptors with both aggressive bladder carcinoma and poor outcome (8); ii) importance of interactions among molecules in the TGF-β1 signaling for the progression of bladder cancer (9); iii) the potential therapeutic use of TGF-β1 in bladder carcinoma (10); and iv) the role of TGF-β1 and its receptor in immune escape in bladder cancer (11).

TGF-β1 is a pleiotropic cytokine, physiologically involved in the proliferation and differentiation of cells, embryonic development, angiogenesis, wound healing and immune regulation (12). Under malignant conditions, TGF-β1 however is considered as a major modulator of tumor behavior. During the initiation and early stage of tumor development, TGF-β1 may serve as a tumor suppressor by inhibiting proliferation and accelerating apoptosis; later on, TGF-β1 becomes a protumor factor by favoring tumor migration, invasion, angiogenesis and immune evasion (13,14). In the latter mode, tumor cells organize strategies to overcome TGF-β1-mediated growth arrest by downregulating or mutating receptors or other means so that they cannot be targeted (15-22).

There are three TGF-β receptors: TGF-βR type I, II and III, respectively. TGF-βRIII has a very short cytoplasmic tail and lacks any signaling motif, whereas TGF-βRI and II are serine/threonine kinases that are essential part of TGF-β1

signal transduction (12,13). TGF-β receptor downregulation and/or mutation in various tumor types, including human bladder cancer, are well covered and discussed in the literature (14-19,23). Therefore, the role of abnormal TGF-β receptors appears to render malignant cells resistant to TGF-β-mediated adverse effects.

In contrast to the loss or mutation of its receptors, TGF-β1 usually is highly expressed by tumor cells (14). Mechanisms of tumor immune evasion, including those summarized below, might be helpful to explain this phenomenon: i) TGF-β1 is capable of inducing regulatory T cells (24); ii) TGF-β1 impairs tumor antigen presentation by inhibiting the maturation of dendritic cells (24); and iii) TGF-β1 'educates' macrophages and fibroblasts to become tumor-associated macrophages or fibroblasts (23,24). None of these mechanisms precludes tumor cells from directly utilizing TGF-β1 signaling through certain alternative mechanism, to promote tumor cell invasion and metastasis, without inhibiting tumor cell proliferation (14,23). We provide evidence that bladder cancer cells exploit mutated TGF-β receptor for TGF-β signal transduction, leading to their enhanced migration and invasion as well as avoidance of growth arrest.

Materials and methods

Ethics statement. The study protocol was approved by the Medical Ethics Committee of Tongji Medical College and performed according to the declaration of Helsinki. All patients gave their written informed consent before participating in this study. The University of Padova and the Thomas Jefferson University Institutes' ethics regulations on research conducted on human tissues were followed.

Human cell line. Cell lines T24, ScaBER and BIU-87 (bladder cancer); PC-3 and DU145 (prostate cancer); A549 (lung cancer); HeLa (cervical cancer); AGS (gastric cancer); A375 and A875 (melanoma); HepG2 and SMMC-7721 (hepatocarcinoma); MDA-MB-435, MDA-MB-231 and MCF-7 (breast cancer); Raji and K562 (leukemia); and L02 (embryo hepatocyte derived) were involved in this study. BIU-87, A875, MDA-MB-435, SMMC-7721, Raji, K562 and L02 were purchased from China Center for Type Culture Collection (CCTCC, Wuhan, China). T24, ScaBER, PC-3, DU145, A549, HeLa, AGS, A375, HepG2, MDA-MB-231 and MCF-7 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Carlsbad, CA, USA) supplemented with 100 ml/l fetal bovine serum (HyClone, Logan, UT, USA) at 37˚C in a humidified atmosphere containing 5% CO_2.

Plasmids and transfections. Plasmids used in this study included pIRES2-EGFP-TβRII-Fc (TβRII-Fc) and pIRES2-EGFP (control). Those interested in further details are kindly referred to the following citation (25). Plasmids were transfected into L02, T24 or ScaBER cell line using Lipofectamine Plus reagent, according to the manufacturer's (Invitrogen, Carlsbad, CA, USA) instruction.

siRNAs, siRNA of Smad2 and Smad3 and their controls, on the other hand, were purchased from RiboBio Company (Guangzhou, China), and transfected into cells also using Lipofectamine Plus reagent, according to the manufacturer's instruction. Forty-eight hours after the transfections, the cells were sorted for the detection of GFP expression or other experiments.

Real-time PCR analysis. Total RNA was extracted from cells with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. For real-time RT-PCR assays, the cDNA sequences of genes were retrieved from NCBI database. The primers were designed with the Oligo Primer Analysis 4.0 software and the sequences were blasted (http://www.ncbi.nlm.nih.gov/BLAST/). Real-time RT-PCR was done as described previously (26). The mRNA level of the detected gene was expressed as the relative level to that of β-actin. The sequences of the primers were as follows: β-actin, 5'-CCTAGAAGCATTTGCGGTGG-3' (sense) and 5'-GAGCTACGAGCTGCCTGACG-3' (antisense); TGF-β1, 5'-ACTACTACGCCAAGGAGGTCAC-3' (sense) and 5'-GAGCAACACGGGTTCAGGT-3' (antisense); TGF-βRII, 5'-AGACGGCTCCCTAAACACTAC-3' (sense) and 5'-GAATGCTCTATGTCACCCACTC-3' (antisense); TGF-βRI, 5'-GACAACGTCAGGTTCTGGCTCA-3' (sense) and 5'-ATCGACCTTTGCCAATGCTTTC-3' (antisense); p15, 5'-GGCAGACAGGTTTAGCTGTTTCATG-3' (sense) and 5'-CCACAATGGAGCTAGAAGCAGGA-3' (antisense); CUTL1, 5'-AAAGACCAGCCTGAAAGTCGG-3' (sense) and 5'-CCAGGGATGAGCTGAAAAAGT-3' (antisense); and Cdc25A, 5'-CTCCTCCGAGTCAACAGATTCA-3' (sense) and 5'-CAGCCACGAGATACAGGTCTTA-3' (antisense).

Patient samples. A total of 46 clinical bladder cancer specimens were consecutively acquired during the period of October 2009 to October 2010, with approval by the Ethics Committee of the Medical Faculty of Tongji Medical College, by transurethral resection or radical cystectomy from untreated cancer patients (Table I). Informed consent was obtained in accordance with the Declaration of Helsinki from all subjects. Data on the patients' clinical and pathologic states were collected, including sex, age, tumor size, pathologic T stage, tumor grade and multiplicity. The pathologic stage of bladder cancer was assessed according to the 2002 UICC TNM tumor stage classification by: i) the superficial bladder cancer (T1) includes pTa and pT1 tumors; and ii) the muscle invasive bladder cancer (T2) includes pT2, pT3 and pT4. Tumor grade was assessed according to the 2004 WHO/International Society of Urologic Pathology grading classification by: i) the well differentiated papillary urothelial neoplasm includes low malignant potential and low grade tumor (low); and ii) the poorly differentiated papillary urothelial neoplasm which includes high grade bladder cancer (high).

Isolation of human primary bladder cancer cells. Fresh bladder cancer specimens were used for tumor cell isolation as described previously (27). Briefly, tumor tissue was washed three times in cold DMEM medium containing 1% FBS and digested with hyaluronidase (Sigma-Aldrich, St. Louis, MO, USA), collagenase and DNase for 1 h at 37˚C. After grinding with semifrosted slides and lysis of RBC, the dissociated cells were incubated on ice for 20 min and then spun down at 500 rpm for 1 min. This process was repeated twice and the cells were first incubated for

Table I. Patient characteristics.

Characteristic	Patients (n)
Sex	
Male	40 (87.0)
Female	6 (13.0)
Age (year)	
<45	7 (15.2)
≥45	39 (84.8)
Tumor size (cm)	
<3	35 (76.1)
≥3	11 (23.9)
Multiplicity	
Single	32 (69.6)
Multiple	14 (30.4)
T stage	
T1	19 (41.3)
T2	27 (58.7)
Tumor grade	
Low	23 (50.0)
High	23 (50.0)
Expression type of TGF-βRII	
Non-point mutation	28 (61.9)
Mutation-free	8 (17.4)
Undetectable	9 (19.6)
Frame-shift	11 (24.0)
Point mutation	18 (39.1)

Data in parentheses are percentages.

2 h to get rid of adhesive cells. Tumor cells were then cultured in DMEM supplemented with 10% FBS, 2 mmol/l L-glutamine, 1.0 mmol/l sodium pyruvate, 100 U/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate in 6-well plate in a humidified incubator at 37˚C with 5% CO_2.

DNA sequencing. Total RNA, extracted from cell lines or primary bladder cancer cells, was reversely transcribed to cDNA. The latter was used to amplify the whole coding sequence of TGF-βRII by PCR with primers (5'-CTGGAAGATGCTGCTTCTC-3' and 5'-ACTGCTCTGAAGTGTTCTGC-3'). PCR products were sequenced by Sangon Biotech (Shanghai, China).

Flow cytometry analysis. Cells were incubated with 1 μg of human IgG/10^5 cells for 15 min at room temperature prior to staining, and then stained with phycoerythrin-conjugated anti-human TGF-β1 or TGF-βRII antibodies and their isotypes (eBioscience, San Diego, CA, USA). The stained cells were used for flow cytometric analysis (BD Biosciences, LSR II).

Immunohistochemistry. To detect TGF-βRII protein, cells were grown in glass slides overnight. After washing twice, slides were fixed in dry acetone for 10 min at room temperature and air-dried for another 10 min. Rabbit anti-human TGF-βRII primary antibody (Millipore), biotinylated goat anti-rabbit IgG, and streptavidin-conjugated horseradish peroxidase (eBioscience) were used for immunohistochemical staining.

Cell proliferation assays. Cells were incubated with PKH-26 dye for membrane staining, and then cultured in the presence or absence of TGF-β1 (2 ng/ml, PeproTech) in 24-well plate, and 24 to 48 h later, the proliferation was analyzed by flow cytometry and expressed with stimulation index (SI).

Cell migration and invasion. Bladder cancer cell motility and invasion were evaluated by Transwell assay as described previously (28). In migration or invasion experiment, cells were allowed to reach confluence in serum-containing complete growth medium and then incubated for 16 h in serum-free medium before treatment of TGF-β1 or TβRII-Fc and smad2+3 siRNA transfection. Matrigel invasion assay was performed in Transwell plates with polycarbonate membrane filters (Corning, Corning, NY, USA). Precoated filters (6.5 mm in diameter, 8 μm pore size, Matrigel 100 μg/cm^2) were rehydrated with 0.1 ml medium. Then, $2x10^5$ pretreated cells in 0.2 ml DMEM were added to the top chamber. Medium (0.6 ml) supplemented with 20% fetal bovine serum was added to each well of the plate to act as a chemoattractant in the lower chamber. Following incubation for 18 h at 37˚C, non-invading cells at the upper surface of the filter were wiped off with a cotton swab, and the invading cells at the lower surface of the filter were fixed for 5 min in 100% methanol and stained with hematoxylin and eosin. Cells that moved through the insert were counted in five random fields and expressed as the average number of cells per field. Experiments were repeated in triplicate. Transwell migration assays were done using the same procedure but without coating filters with Matrigel.

Co-immunoprecipitation assay. Co-immunoprecipitation was performed as described previously (29). Briefly, cells were harvested at 0, 5, 10 and 20 min following TGF-β1 (2 ng/ml) treatment. Cell extracts were first precleared with 25 μl of protein A-agarose (Sigma-Aldrich). The supernatants were immunoprecipitated with anti-TGF-βRII antibody for 1 h at 4˚C, followed by incubation with protein A-agarose overnight at 4˚C. The complexes were collected by centrifugation for western blot analysis.

Western blot analysis. Cell lysates and prestained m.w. markers were separated by SDS-PAGE followed by transfer onto nitrocellulose membranes. The membranes were blocked in TBST (Tris-buffered saline with 0.5% of Triton X-100) containing 5% non-fat milk or BSA, and probed with primary antibodies (R&D Systems). After incubation with the secondary Ab conjugated with HRP, membranes were extensively washed, and the immunoreactivity was visualized by ECL according to the manufacturer's protocol (ECL kit, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Antibodies smad2/3, psmad2/3, β-actin, p15, CUTL1 and Cdc25A were purchased from Santa Cruz Biotechnology.

Statistical analysis. Results were expressed as mean values ± SD and interpreted by ANOVA or χ^2 test (in statis-

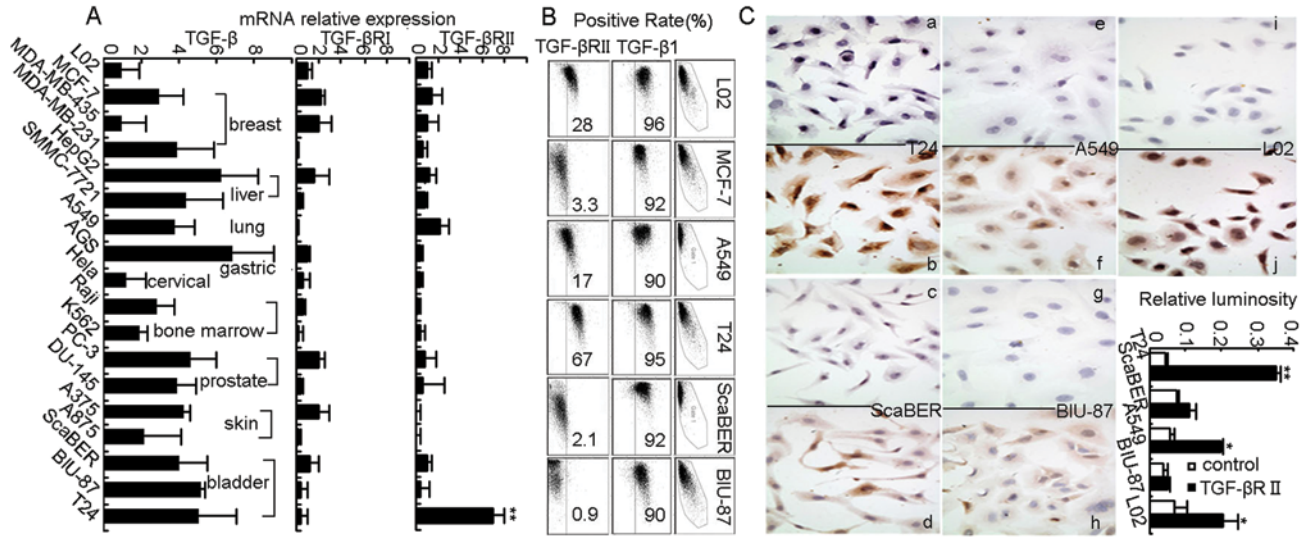

Figure 1. TGF-βRII is highly expressed in human bladder cancer cell line T24. (A) qRT-PCR showed expression of TGF-β1, TGF-βRI and TGF-βRII in different kinds of human cancer cell lines, **P<0.01 for each cancer cell line compared to embryo hepatocyte derived L02 cells. (B) Flow cytometry showed expression of cytoplasmic TGF-β1 and membraned TGF-βRII in human cancer cell lines. (C) Cell immunohistochemistry (a, c, e, g and i, isotype control; and b, d, f, h and j, experimental group) also showed strong staining of TGF-βRII in T24 cell membrane, *P<0.05, **P<0.01 compared to control group. Magnification, x400. Similar results were obtained at least in three separate experiments.

tics of patient sample, we used χ^2 continuity correction when 1≤T<5). SPSS version 12.0 (Chicago, IL, USA) was used for statistical analysis. Differences were considered to be statistically significant when P<0.05.

Results

TGF-βRII is highly expressed in bladder cancer cell line T24. This study first examined TGF-β1 expression and its receptors TGF-βRI and TGF-βRII by real-time RT-PCR in human tumor cell lines derived from a variety of tissues, including breast, liver, lung, gastric, prostate, skin, bladder and bone marrow. As expected, all the tumor cell lines were shown to highly express TGF-β1 but to have low expression of TGF-βRI (Fig. 1A), which was consistent with previous reports (14-16,20-23). Unexpectedly however, we found that although TGF-βRII was weakly expressed in most tumor cell lines but strongly expressed in T24 bladder cancer cells (Fig. 1A). Such abnormal high expression of TGF-βRII in T24 cells was further confirmed by flow cytomety and cellular immunohistochemical staining (Fig. 1B and C). Thus, bladder cancer cell line T24 was identified as highly expressing TGF-βRII.

TGF-βRII engagement does not induce growth arrest but strengthens TGF-β1-mediated cell invasion in T24 bladder cancer cells. Next, this study turned to the question of whether TGF-βRII in T24 bladder cancer cells was functional. TGF-β1 impacts on cell growth arrest are well known in the literature (12-14). First we performed the *in vitro* proliferation assay, and found that the addition of TGF-β1 effectively inhibited the growth of normal liver cell line L02 and the bladder cancer cell line ScaBER, but not T24 cell growth (Fig. 2A). On the other hand, an *in vitro* Transwell assay found that the addition of TGF-β1 markedly promoted the migration and invasion of T24 cells (Fig. 2B, C and D). To test and confirm these initial results, a comparable approach was used to transfect TGF-βRII-Fc vector or smad2/3 siRNA, resulting in the blocking of TGF-βRII or silencing smad2/3, the downstream molecules of TGF-βR signaling (Fig. 2E). Under such conditions, we found that the above effects of TGF-β on T24 cells were relieved (Fig. 2A, B, C and D). These findings suggest that TGF-βRII does not mediate T24 cell growth arrest but is capable of promoting T24 cell migration and invasion through the TGF-β signal pathway.

TGF-βRII signaling fails to regulate proliferation-associated p15 and Cdc25A expression but enhances invasiveness-associated CUTL1 expression in T24 cells. Having identified a vital role for TGF-βRII in cell migration and invasiveness, this study turned next to explore the possible molecular mechanism through which TGF-β1 binding TGF-βRII resulted in the failure of growth arrest but the enhancement of motility in T24 bladder cancer cells. The literature has already identified the mechanisms of TGF-β1-mediated growth arrest which involve upregulating cyclin-dependent kinase (CDK) inhibitor p15 and downregulating CDK4/6-activating phosphatase Cdc25A (30-32). Looking further, this study found that TGF-β1 stimulation was capable of upregulating p15 and downregulating Cdc25A in both ScaBER and L02 cell lines without any undue effect on the expression of p15 and Cdc25A in T24 cell line, evaluated by both RT-PCR and real-time PCR (Fig. 3A and B). Consistently, p15 and Cdc25A proteins in T24 cells were not affected by TGF-β stimulation, evaluated by a western blot analysis (Fig. 3C). On the other hand, homeobox transcription factor CUTL1 has been well demonstrated as a critical target of TGF-β1 signaling to mediate the promotion of cancer cell motility and invasiveness (33-35). Therefore, besides p15 and Cdc25A, this study was able to also determine the expression of CUTL1. Interestingly, the expression of CUTL1 was significantly upregulated by the addition of TGF-β1 in T24 cells but not in L02 or ScaBER cells (Fig. 3A,

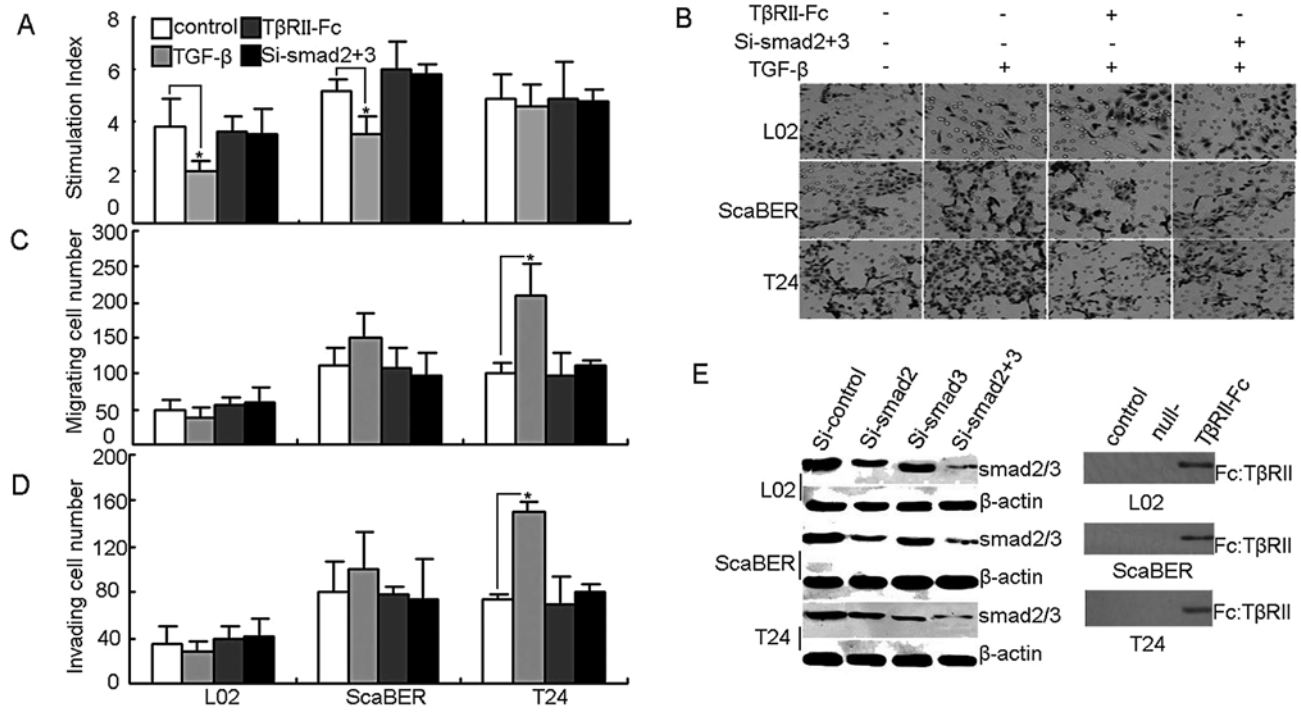

Figure 2. TGF-βRII-transduced TGF-β1 signaling does not induce cell growth arrest but promotes migration and invasion in T24 cell. (A) Cell proliferation assays were performed in human L02, ScaBER and T24 cell line. Each group was treated with 2 ng/ml TGF-β1 for 48 h, or transfected with TβRII-Fc or smad2+3 siRNA for 48 h, stimulation index was detected by flow cytometry (FCM). (B, C and D) Transwell assay showed distinct ability of motility in three kinds of cells. Cell lines were loaded into the top chamber of Transwell filter units with or without TGF-β1 (2 ng/ml) stimulation and allowed to migrate or invade using 20% fetal bovine serum in the lower chamber. Non-migrating cells were removed from the upper surface of the Transwell filter, and migrating cells on the lower surface were stained and quantified. Magnification, x100. (E) The left panel shows western blot analysis silencing Smad2/3 with siRNA transfection. Smad2 and Smad3 siRNAs were both separately and mixed-transfected into cultured cells, and Smad2/3 were effectively silenced in combination after 48 h. The right panel shows the protein levels of TβRII-Fc in the supernatants. Forty-eight hours after TβRII-Fc transfection, the spernatants were collected and analyzed by western blot analysis. Data were obtained from three independent experiments having four replicates per condition. *P<0.05, experiment group vs. control group.

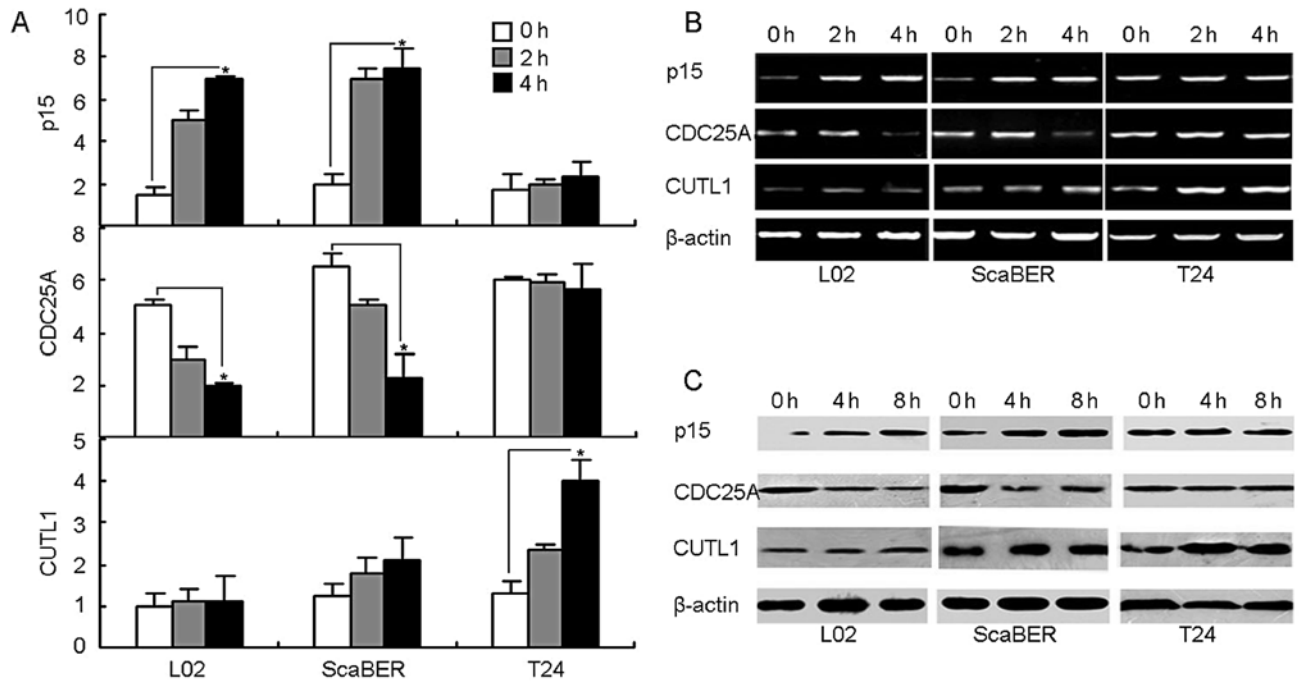

Figure 3. TGF-β1/TGF-βRII signaling has no effect on p15 and CDC25A expression but upregulates CUTL1 in T24 cells. (A) qRT-PCR mRNA levels of p15, CDC25A and CUTL1 in L02, ScaBER and T24 treated with 2 ng/ml TGF-β1 for 0, 2 and 4 h. *P<0.05 for each TGF-β1 treated group versus non-treated group. (B) The expression of p15, CDC25A and CUTL1 was detected by conventional RT-PCR after 2 or 4 h treatment with TGF-β1 (2 ng/ml). (C) Western blot analysis of TGF-β stimulation induced different changes of p15, CDC25A and CUTL1 in L02, ScaBER and T24. No variation of p15 and CDC25A but obvious elevation level of CUTL1 expressed in T24 after TGF-β1 stimulation for 4 and 8 h. Data were obtained from experiments performed in triplicate.

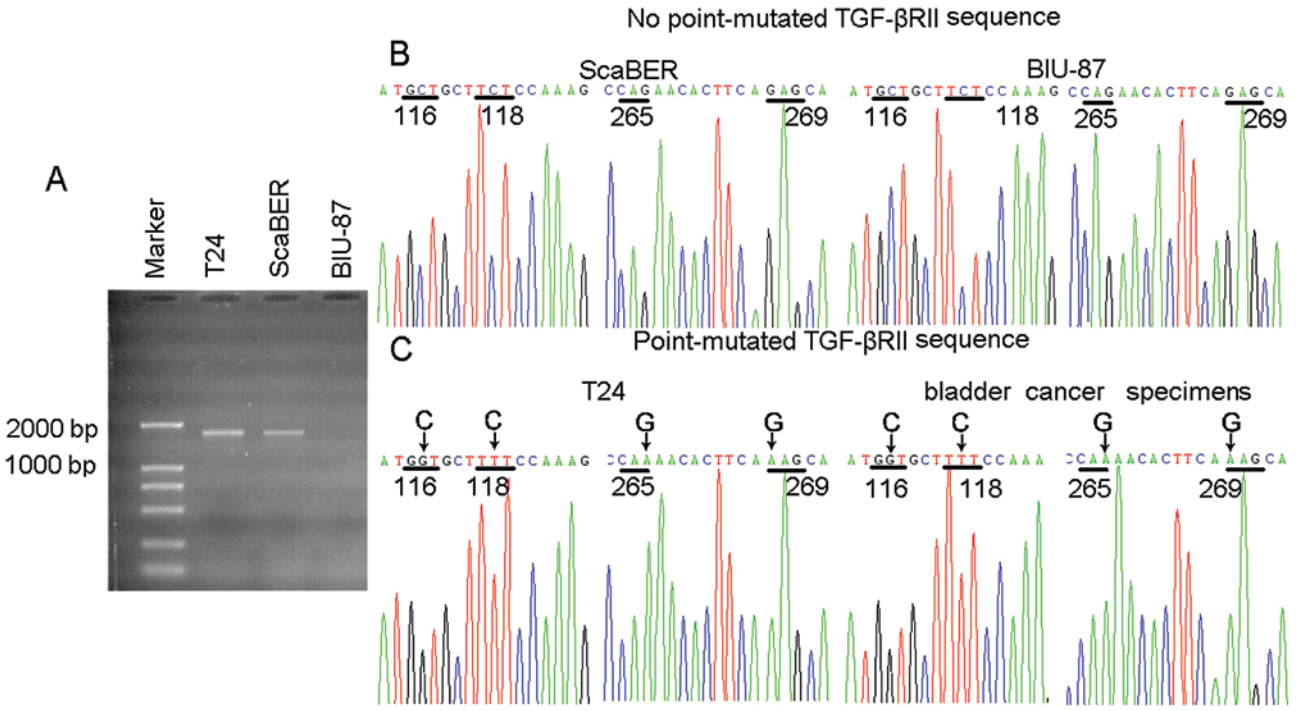

Figure 4. Sequencing analysis of TGF-βRII mutation in T24 cells and bladder cancer specimens. (A) Aagarose gel electrophoresis showed the product of TGF-βRII by PCR from three bladder cancer cell lines. Visible PCR products are apparent in lane 2 and 3. (B) The sequencing analysis showed no TGF-βRII mutation in ScaBER or BIU-87 cell line. (C) The sequencing analysis showed TGF-βRII point mutations in T24 and primary bladder cancer cells.

B and C). Therefore, in T24 bladder cancer cells, the TGF-βRII signaling pathway is ineffective in the regulation of p15 and Cdc25A expression but highly efficacious in the regulation of CUTL1 expression, leading to the evasion of growth arrest and the enhancement of motility and invasion.

Sequencing analysis of TGF-βRII mutation in T24 cell line. Further investigation was necessary into the molecular basis by which TGF-β1 signaling had no effect on p15 and Cdc25A expression but was efficacious in the upregulation of CUTL1 in T24 cells. In bulk tumor cell population the growth of TGF-βR^{high} tumor cells is declined due to TGF-β1 signaling-mediated growth arrest, leading, in turn, to the domination of TGF-βR$^{low/neg}$ tumor cells, depending on the type of cultured cells involved. Paradoxically, T24 bladder cancer cell line maintains the high expression of TGF-βRII. To explain this, it was reasonable to speculate that T24 cells employed a mutation strategy to encrouch TGF-β signaling. In this regard, a pair of primers was designed to amplify 1707 bp cDNA fragment covering the coding region of TGF-βRII. By 25 cycles, the visible PCR products from T24 and ScaBER cell lines were confirmed in an agarose gel (Fig. 4A). Further sequencing analysis indicated that TGF-βRII was not mutated in either ScaBER or BIU-87, but mutated with several single nucleotides in T24 (Fig. 4B and C). Notably, one point mutation was the GAG to AAG transition, leading to corresponding amino acid transition of Glu^{269}→Lys in the cytoplasmic domain of TGF-βRII. Glu and Lys belong to acidic and basic amino acids, respectively. Thus, this Glu^{269}→Lys mutation might profoundly change the electric property of TGF-βRII, leading to altered conformations and abnormal signaling pathways.

Mutated TGF-βRII-transduced signaling is Smad2/3 dependent in T24 cells. The fact, that Smad2/3 are the classical downstream molecules for TGF-β signaling transduction (23,24), begged the question of whether Smad2/3 were also required for the mutated TGF-βRII signaling pathway in T24 cells. This, in turn, first called for the measurement of the active form of Smad2/3 by measuring its phosphorylation state by western blot analysis. It was found that the phosphorylation was induced 30 min after the stimulation of 2 ng/ml TGF-β1 in L02 and ScaBER cells as well as T24 cells (Fig. 5A), suggesting that the mutated TGF-βRII signaling transduction might be through the classical Smad2/3 pathway. Moreover, by pulling down the TGF-βRII complex with anti-TGF-βRII antibody, it was observed that Smad2/3 proteins were bound to the complex (Fig. 5B), suggesting the interaction of Smad2/3 and TGF-βRII in T24 cells. When silencing Smad2 and Smad3 in T24, the binding complex can not be detected with additional TGF-β1, expression of CUTL1 then having almost no change (Fig. 5C), indicating that elevated level of CUTL1 was Smad2/3 dependent in T24 cells. Taken together, these findings suggested that the transduction of TGF-β signaling by mutated TGF-βRII is Smad2/3-dependent in bladder cancer T24 cell line.

GAG→AAG mutation of TGF-βRII occurs in bladder cancer patients and is correlated to high aggressiveness. To translate the implications of the above *in vitro* data *in vivo*, the sequence of TGF-βRII cDNA in bladder cancer patients was further analyzed. Primary bladder cancer cells were isolated from fresh specimens of bladder cancer patients (n=46, Table I), and then used for RT-PCR to amplify the TGF-βRII cDNA (25 cycles) for sequencing. As expected, similar mutation patterns were

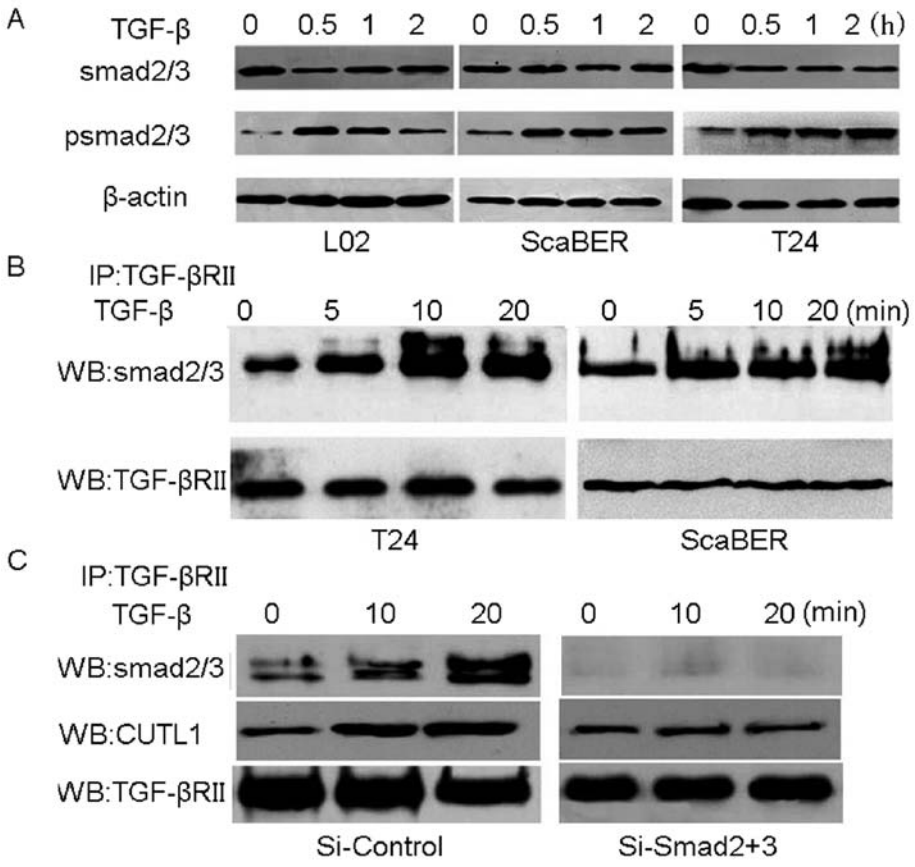

Figure 5. Mutated TGF-βRII transduces Smad2/3-dependent pathway in T24 cells. (A) Smad2/3 phosphorylation was indicated by western blot analysis. After treating with 2 ng/ml TGF-β1, the phosphorylation of Smad2/3 was detected in cell lines. (B) Smad2/3 proteins were bound to TGF-βRII complex in T24 and ScaBER cell lines. Cells were treated with 2 ng/ml TGF-β1 for 0, 5, 10 and 20 min, co-immunoprecipitation showed the interaction of Smad2/3 and TGF-βRII in T24 cells. (C) Co-immunoprecipitation showed after TGF-β1 treament, no binding product appeared in Si-Smad2+3 group, the following western blot analysis test showed level of CUTL1 had almost no change after Smad2 and Smad3 silencing.

found in primary bladder cancer cells as in T24 cells. A total of 18 specimens showed GAG→AAG mutation (39.1%), which was also companied by other mutations observed in T24 cells (Fig. 4C). Therefore, it is safe to say that GAG→AAG mutation of TGF-βRII also occurs in bladder cancer patients. To explore the possible clinical significance of such point mutations, patients were subdivided into 2 groups: non-mutated TGF-βRII (group 1, n=28) and point-mutated TGF-βRII (group 2, n=18). In cases of bladder cancer, tumor grade is used to reflect the relapse and metastasis, and pathologic T stage, however, may reflect the degree of infiltration and invasion by tumor cells. By comparing the clinical and pathologic features of 2 groups, significant differences were found in both pathologic T stage and tumor grade stages ($P<0.01$; Table II). Together, these findings suggested that GAG→AAG mutation of TGF-βRII may also occur in patients with bladder cancer and such TGF-βRII mutations seem to be correlated with worse malignant phenotypes and poor prognosis.

Mutated TGF-βRII transduces TGF-β1 signaling to modulate the behavior of primary bladder cancer cells. The mutation-induced relief from growth arrest and strengthened motility in T24 bladder cancer cell line TGF-βRII were found to be similarly at work in primary bladder cancer cells. On the basis of the sequencing results, the primary bladder cancer cells from 12 patients were classified into two groups: no TGF-βRII mutation (n=6) and mutation with GAG→AAG (n=6). The expression of TGF-βRII between these two groups was then compared. The immunohistochemical staining showed that TGF-βRII protein was strongly expressed in the mutation with GAG→AAG group but not in the no mutation group (Fig. 6A). As a parallel observation, the primary bladder cancer cell growth was not affected by TGF-β treatments in the mutation group (Fig. 6B). On the other hand, TGF-β treatment enhanced the invasion of these cells from GAG→AAG group, which were impaired by TGF-βRII blockade (Fig. 6B). Measurements, furthermore, of the expression of p15, Cdc25A and CUTL1 in the cultured primary bladder cancer cells in the presence or absence of TGF-β, as expected, showed that p15 and Cdc25A were not affected, whilst CUTL1 was upregulated by TGF-β in GAG→AAG group (Fig. 6C and D). Therefore, TGF-βRII GAG→AAG mutation in bladder cancer patients might favor cancer cell migration and survival.

Discussion

Tumor cells evolve multiple strategies, including the mutation of TGF-βRII, to overcome TGF-β signaling-mediated growth arrest. Previous studies have reported that TGF-βRII mutations might abolish TGF-β-induced growth inhibition in breast, head and neck, colon and endometrial cancers (16). This study

Table II. Comparison of clinical and pathologic characteristics.

Variable	Patients (n)			
	Non-point-mutated TGF-βRII	Point-mutated TGF-βRII	χ^2 trend	P-value
Pathologic T stage				
T1	16 (57.1)	3 (16.7)	7.404	0.007
T2	12 (42.9)	15 (83.3)		
Tumor grade				
Low	19 (67.9)	4 (22.2)	9.127	0.003
High	9 (32.1)	14 (77.8)		
Tumor size (cm)				
<3	23 (82.1)	12 (66.7)	△0.717	0.397
≥3	5 (17.9)	6 (33.3)		
Age				
<45	4 (14.3)	3 (16.7)	△0.003	0.956
≥45	24 (85.7)	15 (83.3)		
Gender				
Female	5 (17.9)	1 (0.6)	△0.578	0.447
Male	23 (82.1)	17 (94.4)		
Multiplicity				
Single	22 (78.6)	10 (55.6)	2.741	0.098
Multiplicity	6 (21.4)	8 (44.4)		

Data in parentheses are percentages. Data starting with the triangle (△) were obtained by χ^2 continuity correction.

has provided further evidence that bladder cancer cells evolve point mutations in the extracellular and cytoplasmic regions of TGF-βRII, which incapacitate the TGF-β-mediated growth arrest but enhance the tumor-promoting effect of TGF-β in the migration and invasion of bladder cancer cells.

Overexpression of TGF-β in human cancers is a general biological phenomenon and switching the role of TGF-β from a tumor suppressor to a tumor promoter is an important step in malignant development. To accomplish this goal, tumor cells employ a variety of molecular mechanisms to downregulate the expression of TGF-β receptors or simply disable their function by using mutation strategies. In the case of TGF-βRII, for example, truncation, deletion, or decreased expression of TGF-βRII has been detected in a variety of primary tumors and tumor cell lines (15-22). Moreover, mutations in TGF-βRII frequently occur at the coding region of exon 3 with a special sequence called microsatellite-like repeats consisting of a 10-base pair (bp) poly-adenine per repeat. Such mutations are characterized by an insertion/deletion of one or two adenines, leading to a truncated protein that lacks the transmembrane domain and the intracellular serine/threonine kinase domain, and found in a variety of malignancies including colon, gastric, non-small cell lung and biliary tract cancers and glioma (36-40). In addition, point mutations in the kinase domain of TGF-βRII causing defective autophosphorylation have been reported in human head and neck carcinoma cell lines (41). In this study, we further showed that point mutations of TGF-βRII occurred in bladder cancer cell line and patients. Two point mutations were detected in the extracellular region and another two were found in the cytoplasmic serine/threonine kinase domain. For the latter, one is synonymous mutation and the other is missense mutation by Glu^{269}→Lys, thus profoundly changing the proximal charge and subsequently influencing the phosphorylation of TGF-βRI. The TGF-β signaling can still be transduced through classical Smad2/3 pathway, regardless of the potent effect of Glu^{269}→Lys mutation on TGF-βRI, whose activation recruits and phosphorylates Smad2/3. In the present study, although we did not investigate how Glu^{269}→Lys mutation affects the phosphorylation of TGF-βRI, elucidating the underlying molecular mechanism undoubtedly has been useful for our understanding of the significance of TGF-βRII in bladder tumorigenesis.

Bladder cancer is the most and second most common genitourinary neoplasia in China and the USA, respectively, which causes up to 12,000 or more annual deaths. However, to date, the progression of bladder cancer is still not well understood. Previous studies have showed the serum levels of TGF-β1 were significantly elevated in invasive bladder cancer patients and TGF-β1, rather than TGF-β2 or 3 was the predominant isoform in bladder cancer cells at protein as well as mRNA levels (42,43), suggesting that TGF-β1 signaling is involved in the progression of bladder cancer. Hung *et al* explored the molecular profile involving TGF-β signaling pathway in bladder cancer, which emphasized the importance of TGF-β signaling in bladder cancer progression (9). Given the known growth-inhibiting effect induced by TGF-β signaling, how bladder cancer cells escape TGF-β-mediated growth arrest

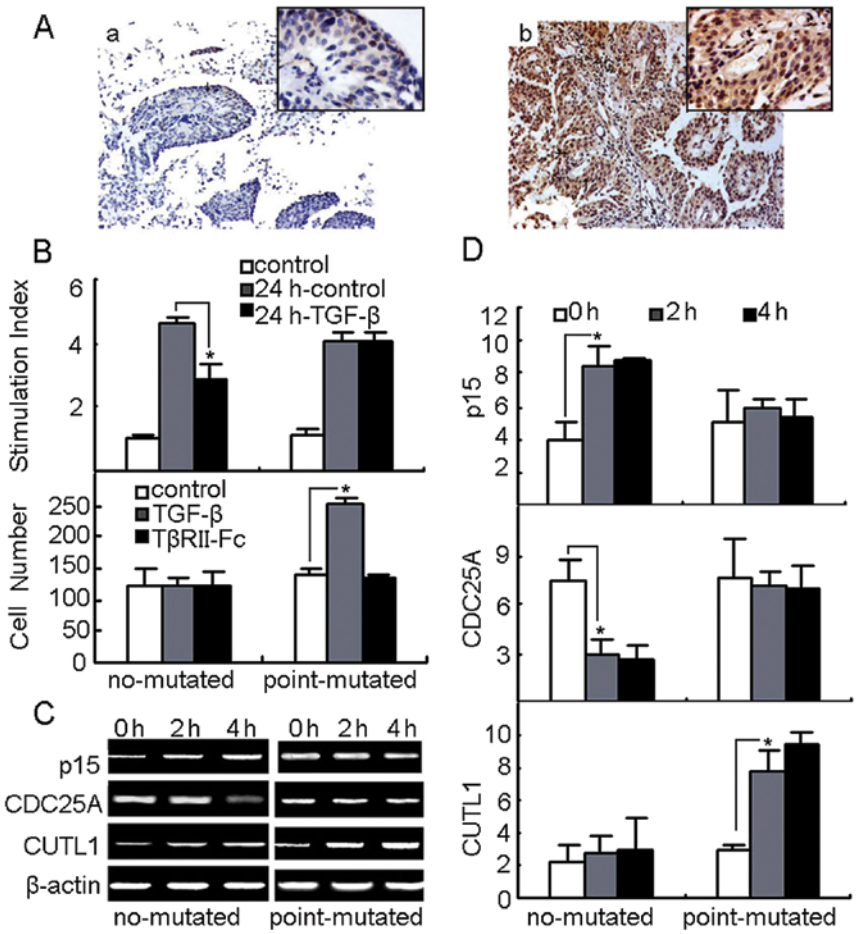

Figure 6. The influence of mutated TGF-βRII-transduced TGF-β1 signaling on the proliferation and invasion of primary bladder cancer cells. (A) Patient bladder cancer specimens were used for TGF-βRII immunohistochemical staining. The result was a representative of slight staining of TGF-βRII in samples without TGF-βRII mutation (a) and strong staining with TGF-βRII mutation (b). Magnification, x100 and x400 in the frames. (B) The influence of TGF-β1 signaling on the proliferation and invasion of primary bladder cancer cells. Single primary bladder cancer cells were isolated from fresh tumor tissues and one proportion of cancer cells were used for mutation determination by sequencing. The other section of the cells were labeled with PKH26 and cultured in the presence of TGF-β1 (2 ng/ml) for 24 h and the proliferation was determined by flow cytometry and shown as stimulation index (top). *P<0.05 compared to 24 h control group. The isolated cancer cells were cultured in the presence of TGF-β1 or TGF-β1 plus TβRII-Fc for 48 h, and then loaded into the Matrigel-precoated top chamber of Transwell filter units. Invading cells were stained with hematoxylin and eosin and counted in five random fields and quantified (bottom). *P<0.05, TGF-β group versus control group. (C and D) The regulation of the expression of p15, CDC25A and CUTL1 in primary bladder cancer cells by TGF-β1 signaling. Single primary bladder cancer cells isolated from fresh tumor tissues and a section of the cancer cells were used for mutation determination by sequencing. The other cells were cultured in the presence of TGF-β1 with different times (0, 2 and 4 h). The total RNA was isolated to determine the gene expression of p15, CDC25A, CUTL1 by (C) conventional and (D) real-time RT-PCR. *P<0.05, experiment group versus control group. Data were obtained from experiments performed in at least triplicate.

still remains elusive. Early studies indicated that TGF-βRII is necessary for TGF-β-mediated growth inhibitory response and TGF-βRII is downregulated in invasive bladder cancer lesions (44). Nevertheless, the increase of TGF-βRII expression was also reported in muscle invasive bladder cancer (45,46). Our present study suggests a reconciliation of this paradox by the mutation of TGF-βRII. Although TGF-βRII seems to be downregulated in most human bladder cancer cell lines as well as other human cancer cell lines, the bladder cancer cell line T24 was found to be capable of overexpressing TGF-βRII (Fig. 1), attributable perhaps to the point mutations of TGF-βRII (Fig. 4). In line with these *in vitro* data, we also found that TGF-βRII was highly expressed in bladder cancer lesions in patients with such point mutations. Thus, by adapting the mutation strategy, bladder cancer cells keep the high expression of TGF-βRII but evade the growth inhibition by TGF-β signaling. Interestingly, this mutation strategy may enhance the intrinsic promoting effect of TGF-β on migration and invasion. This phenomenon was observed in both the bladder cancer cell line and primary tumor cells. In line with our findings, previous studies have showed that a point mutation in TGF-βRII may have failed to restore TGF-β-induced growth arrest but was still able to induce TGF-β-induced migration (47,48). On the basis of the findings here and in other studies, it appears that the mutation of TGF-βRII abrogates the tumor suppressor functions of TGF-β but strengthens other pro-oncogenic effects of TGF-β in bladder cancer.

CUTL1, also known as CDP, Cut, or Cux-1, a homeodomain transcriptional regulator of development and cell cycle progression, and has been identified as a key downstream effector of TGF-β signaling in modulating tumor cell motility and invasion. Michl *et al* found that TGF-β induces CUTL1

expression via Smad4 and p38MAPK and CUTL1 may stabilize Src protein, leading to the activation of Src-regulated downstream signaling molecules such as RhoA, Rac1, Cdc42 and ROCK and subsequent cell mobility and invasion (33,35). This study found that the addition of TGF-β increased the expression of CUTL1 and the point mutations of TGF-βRII, albeit, further augmented such upregulation in bladder cancer cell line T24 and some primary bladder cancer cells. Although the underlying mechanism was not elucidated here, some clues may be drawn from other studies. In addition to the canonical Smad-mediated signaling pathway, other signal molecules may also be integrated and execute TGF-β signaling. It is known that cytoskeleton reorganization is prerequisite for cell mobility and invasion and governed by small guanosine triphosphatases (GTPases) of the Rho/Rac/Cdc42 family. Coincidently, recent studies found the crosstalk between the classical TGF-β/Smad pathway and Rho GTPases and through TGF-β signaling pathway small GTPase molecules could be transcriptionally upregulated and functionally activated (30). Activation of Rho GTPases by binding GTP instead of GDP leads to the interaction with multiple effector proteins, most of which are serine-threonine kinases, such as Rho coiled-coiled kinase (ROCK1), thereby resulting in actin polymerization via the ROCK1/LIMK2/cofilin pathway. In the present study, although the Rho pathway was not determined *per se*, it is logical to assume that the point mutations of TGF-βRII were useful for the activation Rho GTPases such as RhoA. Besides TGF-βRI, TGF-βRII has also been shown to interact with other molecules including cyclin B2, Hsp90, endoglin and AP2B1. Whether TGF-βRII, especially in its mutated form, is capable of interacting with Rho GTPases, promoting bladder cancer cell motility, is nevertheless worthy of verification.

In summary, this study showed that TGF-βRII, by virtue of its extracellular and cytoplasmic point mutations, confers on bladder cancer cells a desensitivity to TGF-β-mediated growth arrest but, at the same time, more ability for TGF-β-promoted motility and migration. These point mutations have potential clinical significance in both prognosis and treatment of patients with bladder cancer.

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