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Original Article

Protein kinase TTK promotes proliferation and migration and mediates epithelial-mesenchymal transition in human bladder cancer cells

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Abstract: Aim: To investigate the expression level of TTK in bladder cancer, and its role in the proliferation and migration. To investigate the relationship between TTK and epithelial-mesenchymal transition (EMT). Patients and Methods: We compared the expression level of TTK between human bladder cancer tissues and normal bladder epithelial tissues from 70 patients using immunohistochemistry, qRT-PCR and western blotting. Subsequently, we conducted cell viability and cell migration experiments to investigate the effect of TTK on bladder cancer cells. Furthermore, we used qRT-PCR to detect the biomarkers of EMT to examine the relationship between TTK and EMT. Results: The expression level of TTK was significantly higher in bladder cancer tissues as compared to the adjacent noncancerous tissues ($P < 0.001$). The qRT-PCR, immunohistochemistry, and western blotting also showed the same trend. Furthermore, cell viability and cell migration assays showed that TTK promoted proliferation and migration of human bladder cancer cells, and mediated EMT. Conclusion: This study showed that high expression of TTK can promote proliferation and migration, and might mediate the EMT process in human bladder cancer cells.

Keywords: Bladder cancer, TTK, proliferation, migration, EMT

Introduction

Bladder cancer is the second most commonly diagnosed genitourinary neoplasm, with approximately 360,000 new cases diagnosed worldwide and 145,000 deaths each year [1, 2]. The majority of bladder cancer (75%) cases are classified as non-muscle-invasive bladder cancer (NMIBC) at the first diagnosis [3], which is characterized by a high possibility of recurrence and progression to muscle invasive bladder cancer (MIBC) [4]. The progression of NMIBC to MIBC is related to epithelial-mesenchymal transition (EMT), which is vital in the proliferation and migration of several cancers [5, 6].

EMT is a complex process in which epithelial cells acquire a mesenchymal phenotype and motility through a cascade of biologic events [7]. During tumorigenesis, changes in EMT regulatory pathways lead to a loss of cellular adhesion, changes in the polarization of the cells and cytoskeleton, detachment, migration, intravasation, and survival in the vascular system; extravasation, and metastasis [8]. Morphologically, EMT is classically characterized by the dedifferentiation from an epithelial to mesenchymal phenotype, marked by the decreased expression of E-cadherin, and increased expression of N-cadherin, vimentin, and cellular proteases [9]. Our previous study showed that EMT could be a critical step in the progression from the pre-invasive to invasive state in bladder cancer [10].

The TTK gene was first identified as a protein kinase in the budding yeast, Saccharomyces cerevisiae [11]. TTK is involved in several aspects of cell-cycle control, including mitotic SAC activation, proper mitotic progression, centrosome duplication, chromosome alignment, error correction of kinetochore-microtubule attachment, and recruitment of SAC components to kinetochores [12-14]. Like many cell-cycle regulators, TTK transcription is deregulated in various human tumors and elevated TTK mRNA

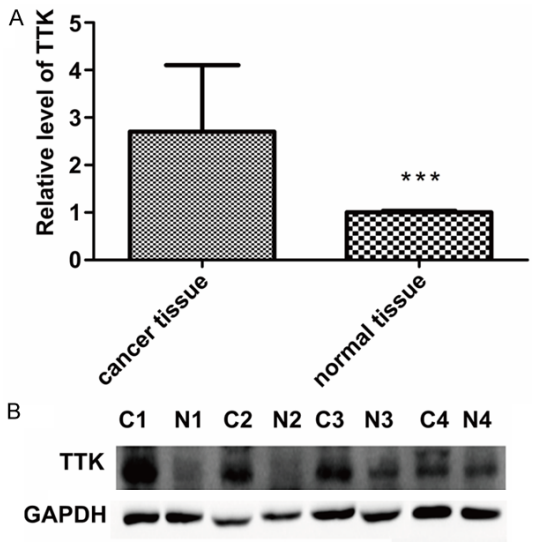

Figure 1. A. TTK expression in bladder cancer tissues and adjacent noncancerous tissues (n = 70) was analyzed by qRT-PCR. The expression of TTK was significantly higher in bladder cancer tissues than in adjacent non-cancer tissues (***, $P < 0.001$). B. TTK marker was assayed in four pairs of bladder cancer and normal tissues by western blotting (C, tumor; N, normal), which showed that TTK was highly expressed in human bladder cancer.

levels were found in several human cancers, including thyroid papillary carcinoma, breast cancer, gastric cancer, bronchogenic carcinoma, and lung cancers [15-17]. Also, high levels of TTK correlated with a more aggressive histologic grade in breast cancers [18]. TTK plays a role in the genotoxic stress response, such as stress caused by DNA damage [19].

In this study, we assessed the differential expression of TTK between bladder cancer tissues and matched adjacent non-cancerous tissues. We also investigated whether TTK promotes proliferation and migration of human bladder cancer cells, and could mediate EMT.

Materials and methods

Patients and tissue specimens

This study was approved by the Ethics Committee of the Second Hospital of Tianjin Medical University. Written informed consent was obtained from the patients for using their tissue specimens. The bladder cancer tissues and adjacent noncancerous tissues were collected from patients who underwent radical cystectomy at the Second Hospital of Tianjin Medical University between 2012 and 2015. All specimens were snap-frozen in liquid nitrogen immediately after resection and stored at -80°C until use. All tissues were independently reviewed by two pathologists to determine the tumor stage and histologic grade according to the 2016 WHO classification of tumors [20].

qRT-PCR analysis

Total RNA was extracted from the tissues according to a previous protocol [21], and reverse-transcribed to cDNA using a Reverse Transcription kit (Roche, USA). Real-time PCR was performed using an Applied Biosystems 7900HT instrument with 20 μl PCR reaction mixture containing 10 μl of 2× Lightcycler 480 SYBR Green I Master (Roche). Reactions were incubated in a 96-well optical plate at 95°C for 10 min, followed by 45 cycles at 95°C for 15 s, and at 60°C for 60 s. Each sample was run in triplicate. A melting curve analysis was conducted to validate the specific generation of the expected PCR product. The primers for amplification of TTK were as follows: 5'-TCCCCAGCGCAGCTTTCTGTAGA-3' (forward) and 5'-CCAGTCCTCTGGGTTGTTTGCCAT-3' (reverse). N-cadherin primers were: forward, 5'-TAAACTGCCTGGCCGAATC-3' and reverse 5'-TCCTTCTGCCTCTATGACCTG-3'. E-cadherin primers were: forward, 5'-CAACGACCCAACCCAAGAA-3' and reverse 5'-CCGAAGAAACAGCAAGAGCA-3'. ZEB1 primers were: forward, 5'-AACTGCTGGCAAGACAAC-3' and reverse 5'-TTGCTGCAGAAATTCTTCCA-3'. Human GAPDH was amplified as an internal control using the forward primer 5'-CTCGCTTCGGCAGCACA-3' and reverse primer 5'-AACGCTTCACGAATTTGCGT-3'. The data were calculated by $2^{-\Delta\Delta}$CT method [22].

Immunohistochemistry and western blotting

TTK expression in tumor tissues was detected by immunohistochemistry and western blotting. Briefly, all tumors were fixed in 4% PFA, embedded in paraffin, and then cut into 5 μm paraffin sections for immunohistochemistry. Deparaffinized sections were dehydrated with alcohol series, and then incubated with a monoclonal antibody, (Abcam, ab187520, rabbit polyclonal antibody, dilution 1:100) at 4°C overnight to detect TTK protein. The protein expression was defined as cytomembrane and/or cytoplasm brown staining (DAB). Slides were then mounted using an aqueous solution and photographed.

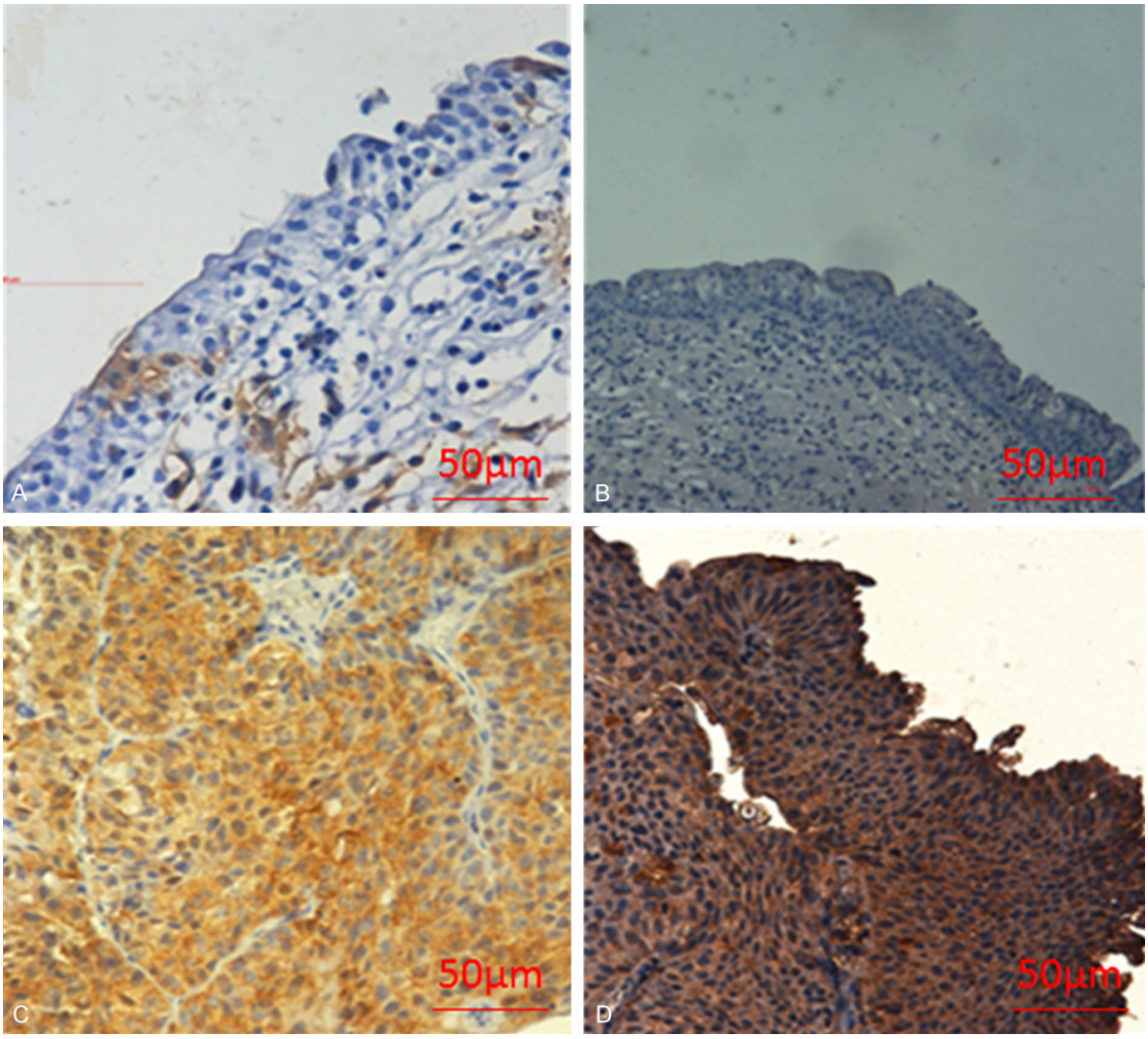

Figure 2. The expression of TTK in normal human bladder urothelium and urothelial carcinoma. (A) and (C) show low expression in normal human urothelium; while (B) and (D) show high expression in human urothelial carcinoma.

Total tissue and cell proteins were extracted using cell lysate buffer with 1% protease/phosphatase inhibitor cocktail. Equal amount of proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat milk in PBS buffer and then incubated with primary antibodies overnight at 4°C. The protein bands were scanned after incubation with secondary antibodies conjugated with ECL and quantitatively measured with Quantity One software.

Cell lines and cell culture

Human bladder cancer cell lines EJ, T24 and 5637 were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS; Gibco, USA) and grown at 37°C in an atmosphere of 95% air and 5% CO_2.

Cell migration assay

Cell migration assay was performed using 24-well Transwells (8 μm pore size; BD Biosciences). Each well of the lower chamber was filled with 500 μl RPMI 1640 (Gibco, USA) containing 10% FBS (Gibco, USA). Human bladder cancer cells (EJ, T24 and 5637) were cultured in serum-free medium overnight. A total of 1×10^5 cells in 500 μl RPMI 1640 containing 1% FBS were plated into each well of the upper chamber. After incubating for 48 h, the cells

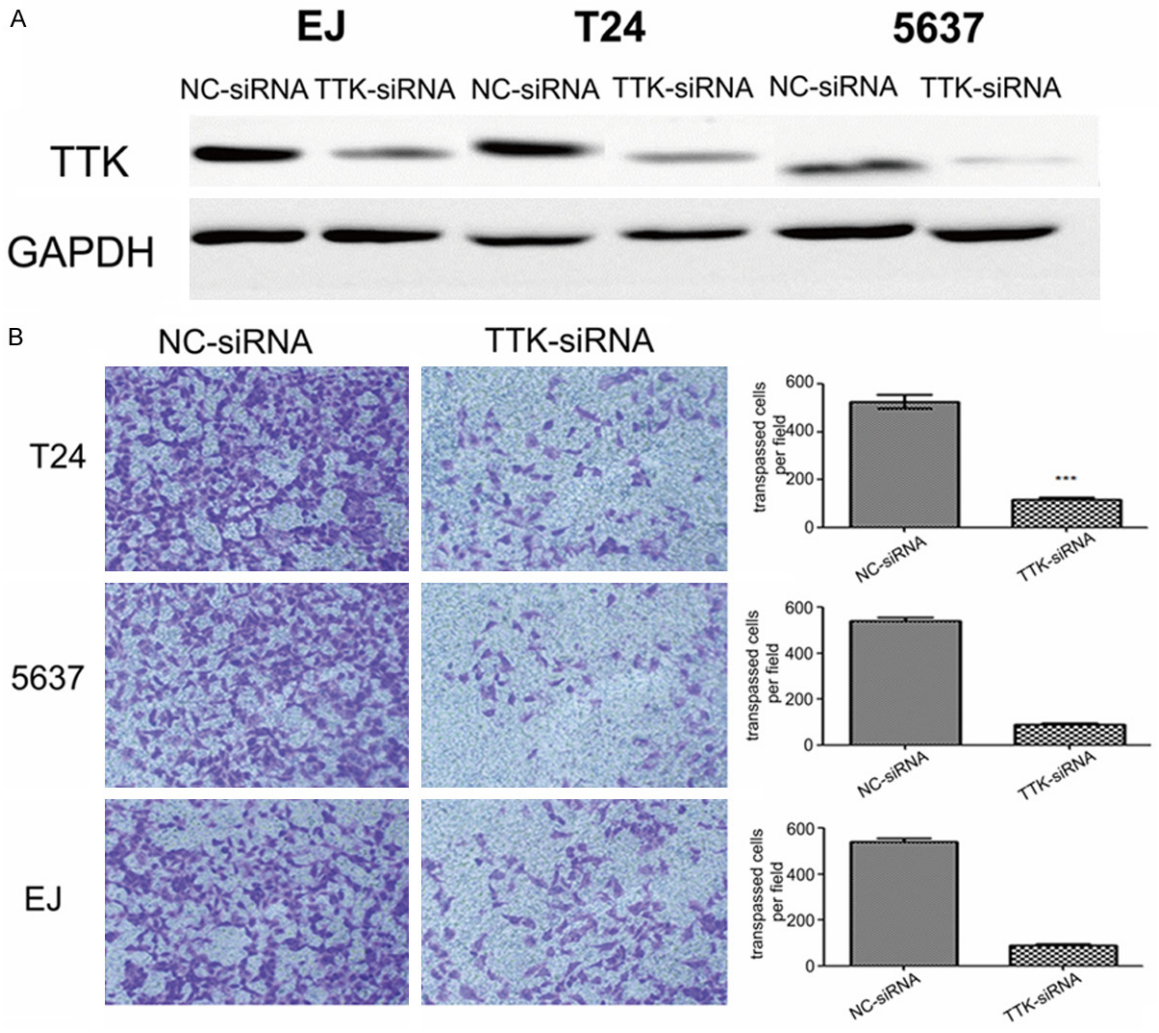

Figure 3. A. Western blotting showed that TTK expression was down-regulated in human bladder cancer cell lines (EJ, T24 and 5637) after transfection with TTK-siRNA for 48 h. B. Images of migration of different human bladder cancer cell lines in transwell assay. The number of migrated cells in the histograms represented mean values per field (from three fields, mean ± SD) (***, P = 0.0013).

were fixed with 4% paraformaldehyde for 15 min and stained with 0.5% crystal violet for at least 30 min. Cells in the upper chamber were removed using cotton swabs. Cells on the lower surface of the membrane were counted and photographed in at least three random microscopic fields (magnification, ×40). The experiments were independently repeated three times.

Cell viability analysis

Bladder cancer cells (EJ, T24 and 5637) were seeded in 96-well plates, with 2.0×10^4 cells per well. The cells were incubated for 0, 24, 48, 72, 96 h. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to analyze the cell viability as previously described [23].

Statistical analysis

Statistical analyses were performed using SPSS Statistical 20.0 software (SPSS Inc., Chicago, IL, USA) and results were considered statistically significant at $P < 0.05$. Data are shown as mean ± SD. The corresponding figures were drawn using GraphPad Prism v5.0 software (GraphPad Software Inc, La Jolla, CA, USA).

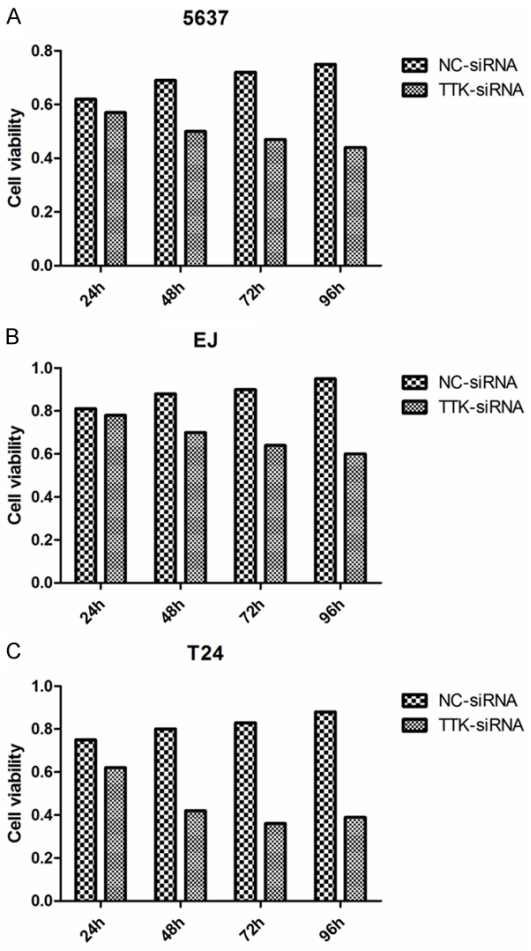

Figure 4. The effects of TTK on cell proliferation. Bladder cancer cell lines were transfected with NC-siRNA and TTK-siRNA for 24, 48, and 72 h. The cell growth was measured by MTT assay. The y-axis represents cell viability based on measured OD560 values. The x-axis represents time course.

Results

TTK expression is increased in human bladder cancer

We first used 70 pairs of bladder cancer tissues and matched adjacent noncancerous tissues to analyze the expression level of TTK by qRT-PCR. The results showed that TTK mRNA expression was higher in the bladder cancer tissues than in the paired adjacent non-tumor specimens (**Figure 1A**, ***, $P < 0.001$).

Next, we conducted immunohistochemistry and western blotting with bladder cancer and normal bladder tissues, and found that the expression of TTK was significantly higher in bladder cancer tissues than in normal bladder tissues (**Figures 1B**, **2**).

Down-regulation of TTK inhibits proliferation and migration of human bladder cancer cells

To examine the effects of TTK on bladder cancer cell growth and proliferation, we conducted siRNA-mediated knockdown experiments in bladder cancer cell lines (T24, 5637 and EJ). The knockdown efficiency of the siRNA was measured by western blotting (**Figure 3A**), with NC-siRNA as a control. The results showed that TTK-siRNA could down-regulate TTK expression. In cell migration with transwell assay, transfection of TTK-siRNA in T24, 5637 and EJ cells significantly decreased cell migration ($P < 0.01$, **Figure 3B**). Furthermore, the TTK-siRNA group showed lower cell viability as compared to the control group ($P < 0.01$, **Figure 4**). These results suggested that TTK contributes to tumorigenesis by promoting growth and migration of bladder cancer cells.

TTK could promote EMT in bladder cancer cells

We compared the expression of EMT biomarkers between the control group and the si-TTK group. N-cadherin and ZEB1 showed significantly lower expression in the si-TTK group as compared to the control group. In contrast, E-cadherin was increased in the si-TTK group ($P < 0.01$, **Figure 5**). These data suggested that TTK may be involved in EMT.

Discussion

Bladder cancer is the most frequently occurring tumor of the urinary tract, and the eighth most common cause of cancer-related deaths in males in the USA in 2017 [24]. The characteristics of bladder cancer are high recurrence rate and poor survival in advanced metastatic stage. Majority of the patients undergo transition from NMIBC to MIBC. The mechanism of disease progression remains unclear, and several studies have shown that it may be mediated by EMT [25]. Mitotic checkpoint gene TTK can play a role in human bladder cancer by mutations and loss of heterozygosis [26], but the definite effect of TTK on bladder cancer remains unknown.

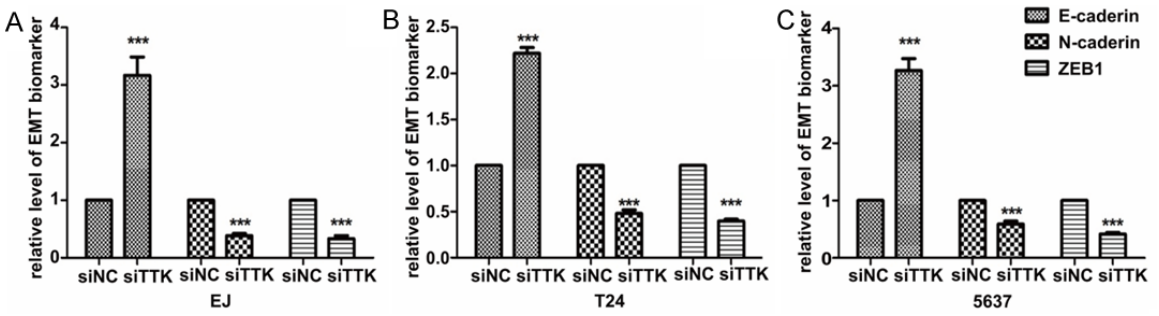

Figure 5. Inhibiting TTK expression in EJ, T24 and 5637 cells decreased the expression of N-cadherin and ZEB1, while the expression of E-cadherin was increased (***, $P < 0.001$).

In this study, we evaluated the expression level of TTK in bladder cancer tissues and normal bladder epithelial tissues. The results showed that TTK had a higher expression in bladder cancer tissues than in normal bladder epithelial tissues. Similar findings have been reported in thyroid papillary carcinoma, breast cancer, gastric cancer, bronchogenic carcinoma, and lung cancers [15-17, 23, 27]. This finding suggested that TTK acts as an oncogene, and could activate some oncogenic signals. Our findings have also shown that down-regulation of TTK could decrease the proliferation and migration of human bladder cancer cells, which could be because TTK is an essential kinase for proper distribution of chromosomes in mitosis, and could cause severe chromosomal segregation defects that lead to cancer cell death [28]. Numerous potent TTK inhibitors have been reported as cancer therapeutic targets in clinical trials [29].

A prominent characteristic of cancer is invasion, and EMT is known to play a role in invasion and metastasis [30-33]. The present study showed that down-regulation of TTK led to decreased expression of N-cadherin and ZEB1, while E-cadherin expression was increased. The results suggested that inhibiting TTK could inhibit the EMT process in human bladder cancers. This is because activation of TTK can promote transforming growth factor-beta (TGF-b)-independent Smad signaling [34], which is one of the primary pathways involved in EMT. Our future study will focus on the molecular mechanism of TTK-mediated EMT in bladder cancer.

In summary, our study showed that TTK was highly expressed in human bladder cancer, and this could promote proliferation, migration, and EMT.

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Disclosure of conflict of interest

None.

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