Thyroid Receptor-Interacting Protein 13 is Correlated with Progression and Poor Prognosis in Bladder Cancer

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Background: Bladder cancer is the fourth most common cancer worldwide. Thyroid receptor-interacting protein 13 (TRIP13) is a member of the AAA+ ATPase family. The upregulation of TRIP13 has been shown to be involved in a few diseases, especially in cancers, but the expression and function of TRIP13 in bladder cancer is still elusive.

Material/Methods: In our study, the expression of TRIP13 was investigated with immunohistochemistry (IHC). The mRNAs of TRIP13 in bladder cancer and adjacent normal tissues were compared using quantitative real-time polymerase chain reaction (qRT-PCR) and IHC scores. The clinical value of TRIP13 was estimated by evaluating its correlation with other clinicopathological factors using the chi-square test. The prognostic significance of TRIP13 was evaluated using univariate and multivariate analyses. The effect of TRIP13 on proliferation and invasion was evaluated using function assays in vitro.

Results: In the 139 samples of bladder cancer tissues, the patients with low and high expression of TRIP13 accounted for 64.03% and 35.97%, respectively. Moreover, the mRNA expression of TRIP13 in bladder cancer was significantly higher than in normal tissues. High expression of TRIP13 was remarkably correlated with T stage, metastasis, and poor prognosis. In addition, TRIP13 was demonstrated to promote the proliferation, invasion, and epithelial-mesenchymal transition (EMT) of bladder cancer.

Conclusions: TRIP13 is correlated with poor prognosis of bladder cancer by promoting proliferation, invasion, and EMT, indicating that TRIP13 may be a promising drug target in bladder cancer.

MeSH Keywords: Cell Proliferation • Epithelial-Mesenchymal Transition • Gallbladder Neoplasms • Mediator Complex Subunit 1 • Neoplasm Invasiveness • Prognosis

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Background

Bladder cancer is the fourth most common cancer and the second most common urologic cancer type [1,2], accounting for about 5% of all new cancers in the United States in 2018 [3]. Approximately 70% of patients with bladder cancer present with non-muscle-invasive disease (NMIBC) with a good prognosis, and the 5-year survival rates are from 80% to 90%. The remaining 30% of patients are diagnosed in a muscle-invasive stage with less favorable prognosis, which may progress to metastatic stage, with a 5-year survival rates of 10% to 15% [4]. In the recent decades, great efforts have been made to improve the survival rates of bladder cancer patients, and great progress has been made. At present, the treatment options for muscle-invasive disease (MIBC) include radical resection followed by standard adjuvant therapy, including chemotherapy and radiotherapy [5]. Moreover, immune checkpoint inhibitors (CPIs) are routinely applied to patients with unresectable tumors [6]. However, most patients with NMIBC still eventually progress to MIBC; therefore, new drugs and novel biomarkers are urgently needed. Moreover, there is no effective biomarker for bladder cancer that can accurately predict patient prognosis and response to different treatments.

Thyroid receptor-interacting protein 13 (TRIP13) is a member of the AAA+ ATPase family, which is known for mechanical forces derived from ATP hydrolysis reactions [7]. TRIP13 influences cells division and proliferation by playing a key role in chromosome recombination, spindle assembly checkpoint, chromosome synopsis, and chromosome structure development during meiosis [7, 8]. Upregulation of TRIP13 has been shown to be involved in certain diseases, especially in cancers. Previous studies reported that TRIP13 was associated with the progression or prognosis of several types of cancers, including hepatocellular carcinoma, colorectal cancer, and lung adenocarcinoma [9–11]. However, the expression and prognostic value of TRIP13 in bladder cancer have not been investigated until now.

In our study, the expression of TRIP13 was investigated with immunohistochemistry (IHC) in 139 bladder cancer patients, and was also detected with qRT-PCR in 9 pairs of fresh bladder cancer tissues and paired normal epithelia. The clinical value of TRIP13 was evaluated by estimating the correlation with clinicopathological factors and survival times. We used in vitro function assay to assess the influence of TRIP13 on proliferation and invasion.

Material and Methods

Patients and follow-up

In Yidu Central Hospital of Weifang and Gansu Provincial Hospital, 384 patients underwent surgical resection of bladder cancer and were diagnosed with bladder cancer by routine pathology from 2007 to 2017, constituting the test cohort. From the test cohort, a total of 139 patients, comprising 100 patients with NMIBC who underwent transurethral tumor resection and 39 patients who underwent radical total bladder cystectomy, were selected as the validation cohort if they had sufficient follow-up and tissues for IHC detection. Moreover, 9 pairs of fresh bladder cancer tissues and paired normal epithelia were collected during surgery and preserved in liquid nitrogen for mRNA extraction. The paraffin-embedded or nitrogen-frozen specimens were obtained with the written consent of patients. This study was approved and supervised by the Ethics Board of Yidu Central Hospital of Weifang and Gansu Provincial Hospital (project 20180904142, dated 2018.10.10).

Cells and reagents

The human bladder cell line TCCSUP was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 medium (Thermo Fisher, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher) and 1% ampicillin-streptomycin. The primary antibody of TRIP13 was purchased from Atlas Antibodies (Bromma, Sweden). The antibodies of the epithelial-mesenchymal transition (EMT) kit, including E-cadherin, N-cadherin, and Snail, were purchased from Cell Signaling Technology (Cat. No. 9782, Cambridge, MA, USA).

Immunohistochemical staining

TRIP13 expression was detected by IHC according to the methods described in a previous study [12]. In brief, the specimens were deparaffinized and rehydrated with xylene and alcohol, and soaked in H2O2 for inactivation of endogenous peroxidase. Following incubation in citrate buffer (pH=6.0) for optimal antigen retrieval, primary antibody of TRIP13 at 1:100 was applied overnight at 4°C. Phosphate-buffered saline was used to rinse the slides, and secondary antibodies (Beyotime, China) were purchased from Cell Signaling Technology (Cat. No. 9782, Cambridge, MA, USA).

The results of IHC were evaluated by IHC scores, which includes the score of staining intensity and positive cell percentage. Staining intensity scores were: 0 for negative staining, 1 for weak staining, 2 for medium staining, and 3 for strong staining. Positive cell percentage scores were: score 1 for <25% of positively stained cells, 2 for 25–50% positive cells, and 3 for more than 50% positive cells. The final IHC score was the product of the score of staining intensity multiplied by the score of positive cell percentage, which ranged from 0 to 9 according...
to our definition. The patients were divided into subgroups by the cut-off of IHC scores, which was determined by receiver operating characteristic (ROC) curve, as described in a previous report [13]. The cut-off point of the cohort was 3.5 in our study, meaning that scores ≥4 were regarded as high expression of TRIP13.

RNA extraction and real-time PCR

TRIzol reagent (Invitrogen, Foster City, CA, USA) was used to extract the total mRNAs from bladder cancer tissues and adjacent normal tissues. SYBR-Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) with the StepOnePlus RT-PCR system (Applied Biosystems) was applied for cDNA synthesis and quantitative PCR. The level of 18S was used as the internal control for normalization for the 2^(-ΔΔCT) equation. The sequences of primers used for real-time PCR experiments were designed as follows:

TRIP13, forward: TGCTGATTGATGAGGTGGAGAG, reverse: GGTTGCACAAGTATCACGCA;
18s, forward, CAGCCACCCGAGATTGAGCA; reverse, TAGTAGCGACGGGCGGTGTG

Proliferation assay

The proliferation of TCCSUP cells was evaluated with MTT assay [14]. In brief, TCCSUP cells were seeded into 96-well plates at 3000 cells per well and cultured for 0 to 60 h. After incubation for indicated times, 50 μg MTT was added per well to incubate cells for 4 h. The supernatants were removed and the crystals at the bottom were re-dissolved by 100 μl DMSO. The optical density at 570 nm (OD570) was measured in a spectrophotometer (Molecular Devices Company, USA) with OD490 as internal control. The readout of OD570 of the control group was defined as the baseline, and the proliferation ratios of other groups were calculated as the ratio to the baseline.

Invasion assay

Tumor invasion of TCCSUP cells was estimated with Matrigel Transwell assay in 8-μm-pore pre-coated Transwells (BD Biosciences, USA) [15]. At 48 h after transfection with siRNA of TRIP13 or scrambled siRNA, TCCSUP cells were seeded into the upper chamber and incubated for 24 h for cell invasion assay. Invaded cells at the bottom were fixed by formalin and stained by 0.05% gentian violet. Cells were counted from at least 5 visual fields under a microscope.

Western blot analysis

Western blot analysis was performed to detect the expression of TRIP13 and the EMT biomarkers. After lysis with RIPA lysis buffer, cells were centrifuged at 12 000 rpm at 4°C for 20 min to decant the pellets and collect the supernatant. Following assessment of protein amounts with Bradford method, 10 μg protein was electrophoresed and transferred into a PVDF membrane (Pall Corporation, Port Washington, MA, USA) with OD490 as internal control. The readout of OD570 of the control group was defined as the baseline, and the proliferation ratios of other groups were calculated as the ratio to the baseline.

Table 1. Basic information of patients with bladder cancer.

| Factors          | Number | Percentage |
|------------------|--------|------------|
| Sex              |        |            |
| Male             | 109    | 78.42%     |
| Female           | 30     | 21.58%     |
| Age              |        |            |
| <60              | 14     | 31.65%     |
| ≥60              | 95     | 68.35%     |
| Tumor diameter (cm) |      |            |
| ≤3               | 70     | 50.36%     |
| >3               | 69     | 49.64%     |
| Tumor number     |        |            |
| Single           | 115    | 82.73%     |
| Multiple         | 24     | 17.27%     |
| pM status        |        |            |
| Negative         | 127    | 91.37%     |
| Positive         | 12     | 8.63%      |
| pT stage         |        |            |
| Ta–T1            | 100    | 71.94%     |
| T2–T4            | 39     | 28.06%     |
| Tumour grade     |        |            |
| Low grade        | 55     | 39.57%     |
| High grade       | 84     | 60.43%     |
| TRIP13           |        |            |
| Low              | 89     | 64.03%     |
| High             | 50     | 35.97%     |

Statistical analysis

SPSS 22.0 software was used to analyze all the data. Chi-square test or Fisher test was used to calculate the correlations between TRIP13 expression and the clinicopathological factors. Kaplan-Meier method was used to show the survival curves, and the log-rank test was used to analyze the differences between subgroups. Cox regression proportional hazards model
was used for multivariate analysis to determine the independent prognostic risks. The t test was used to calculate differences between groups in in vitro experiments. P values less than 0.05 were considered as statistically significant.

**Results**

**TRIP13 was upregulated in bladder cancer**

The expression of TRIP13 in 139 samples of bladder cancer was detected with IHC. The 139 cases included 100 NMIBC and 39 MIBC (Table 1). The expression of TRIP13 was mainly observed in the nucleus, which was consistent with its main function as a spindle composition (Figure 1A). According to the criteria described in the Materials and Methods section above, the patients with bladder cancer were further divided into subgroups with high and low TRIP13 expression, accounting for 64.03% and 35.97%, respectively (Table 1). Moreover, the IHC scores of patients with tumors at T0-T1 and T2-T4 were compared. Patients with T2-T4 tumors had higher TRIP13 expression than the patients in T0-T1 stage. This result suggested that TRIP13 expression in tumors with MIBC stage was higher than in those with NMIBC stage. In addition, the expressions of TRIP13 in bladder cancer and patient-paired normal bladder epithelia were detected with qRT-PCR (Figure 1B). The mRNA expression of TRIP13 in bladder cancer was significantly higher than in normal tissues, indicating that TRIP13 expression was upregulated in bladder cancer.

**TRIP13 expression was associated with tumor infiltration and metastasis**

Using the chi-square test or Fisher test, we further evaluated the correlation between TRIP13 expression and clinicopathological factors (Table 2), showing that high expression of TRIP13 was remarkably correlated with T stage and metastasis. Patients with high TRIP13 expression were more likely
to have advanced T stage and positive metastasis, suggesting that TRIP13 plays an important role in infiltration and invasion of bladder cancer.

**TRIP13 was associated with poor prognosis of bladder cancer**

The prognostic value of TRIP13 was further analyzed by univariate analysis. The survival rates were calculated by the Kaplan-Meier test and statistical differences between subgroups were evaluated by the log-rank test (Table 3). Multiple tumors, positive metastasis, advanced pT stage, and high grade were all notably associated with low survival rates (Figure 2A–2D). Moreover, the expression of TRIP13 was also a prognostic biomarker for bladder cancer \( (P=0.013) \). High TRIP13 was strongly associated with poor prognosis of bladder cancer patients (Figure 2E).

All the significant prognostic biomarkers in the univariate analysis were brought into the Cox-regression hazard model to determine the independent prognostic factors (Table 3). In the multivariate analysis, both pT stage and tumor grade were confirmed as independent prognostic factors (Table 3). However, the statistical significance of TRIP13 as an independent biomarker was not notable \( (P=0.106) \), which was attributed to the intense correlation of TRIP13 with pT stage and metastasis.

**TRIP13 promoted the proliferation, invasion, and EMT of bladder cancer**

Numerous studies have suggested that TRIP13 affects cell proliferation by influencing the mitotic time, which is a key regulator of meiotic recombination and the spindle assembly checkpoint [16]. In our study, the effects of TRIP13 on proliferation were detected with the MTT assay with or without TRIP13 knockdown. TRIP13 expression was first knocked down

| Factors      | TRIP13 expression | p*   |
|--------------|-------------------|------|
|              | Low   | High |      |
| Sex          |       |      |      |
| Male         | 71    | 38   | 0.606|
| Female       | 18    | 12   |      |
| Age          |       |      |      |
| <60          | 26    | 18   | 0.452|
| ≥60          | 63    | 32   |      |
| Tumor diameter (cm) |       |      |      |
| ≤6            | 43    | 27   | 0.524|
| >6            | 46    | 23   |      |
| Tumor number |       |      |      |
| Single        | 76    | 39   | 0.350|
| Multiple      | 13    | 11   |      |
| pM status    |       |      |      |
| Negative      | 86    | 41   | 0.008**|
| Positive      | 33    | 9    |      |
| pT stage     |       |      |      |
| Ta–T1         | 71    | 29   | 0.010|
| T2–T4         | 18    | 21   |      |
| Tumour grade |       |      |      |
| Low grade    | 35    | 20   | 0.938|
| High grade   | 54    | 30   |      |

* Calculated with Chi-square test; ** calculated with Fisher test.
down in the human bladder cancer cell line TCCSUP and verified with Western blotting (Figure 3A). After TRIP13 knockdown, TCCSUP proliferation was significantly decreased after 48-h culture (Figure 3B), which indicated that TRIP13 is required in the proliferation of bladder cancer.

In the clinical analysis, we demonstrated that TRIP13 was correlated with pT stage and metastasis, indicating that TRIP13 may be also involved in tumor invasion in bladder cancer, so we performed Transwell assay to investigate the role of TRIP13 in tumor invasion. We found that invasion of TCCSUP was substantially attenuated after knockdown of TRIP13 (Figure 3C). Since the enhanced EMT was a main cause for tumor invasion, we further detected several EMT biomarkers with Western blotting after silencing TRIP13. Interestingly, the detected biomarkers changed along with TRIP13 downregulation. E-cadherin expression increased significantly while N-cadherin and Snail decreased after silencing TRIP13 (Figure 3D), indicating that TRIP13 is required in the EMT of bladder cancer.

### Table 3. The prognostic value of TRIP13 and other clinicopathological factors.

| Factors                  | Univariate analysis | Multivariate analysis |
|--------------------------|---------------------|-----------------------|
|                          | 5-year survival rate% | P* | HR  | 95% CI | P** |         |
| Sex                      |                     |     |     |       |     |         |
| Male                     | 47.6                | 0.880 |     |       |     |         |
| Female                   | 44.8                |     |     |       |     |         |
| Age                      |                     |     |     |       |     |         |
| <60                      | 50.6                | 0.838 |     |       |     |         |
| ≥60                      | 46.3                |     |     |       |     |         |
| Tumor diameter (cm)      |                     |     |     |       |     |         |
| ≤3                       | 49.6                | 0.490 |     |       |     |         |
| >3                       | 45.2                |     |     |       |     |         |
| Tumor number             |                     |     |     |       |     |         |
| Single                   | 52.1                | 0.002 | 1 | 0.98–5.56 | 0.056 |         |
| Multiple                 | 24.6                |     | 2.33 |       |     |         |
| pM status                |                     |     |     |       |     |         |
| Negative                 | 51.0                | <0.001 | 1 |       |       | 0.24–3.05 | 0.815 |
| Positive                 | 0                   |     | 0.86 |       |     |         |
| pT stage                 |                     |     |     |       |     |         |
| Ta–T1                    | 59.1                | <0.001 | 1 | 1.17–4.68 | 0.016 |         |
| T2–T4                    | 11.3                |     | 2.34 |       |     |         |
| Tumour grade             |                     |     |     |       |     |         |
| Low grade                | 63.9                | 0.005 | 1 | 1.14–4.20 | 0.019 |         |
| High grade               | 31.5                |     | 2.19 |       |     |         |
| TRIP13                   |                     |     |     |       |     |         |
| Low                      | 58.5                | 0.013 | 1 | 0.90–2.95 | 0.106 |         |
| High                     | 28.0                |     | 1.63 |       |     |         |

* Calculated by log-rank test; ** calculated by Cox-regression model.

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Discussion

Bladder cancer is the most common malignancy of the urinary tract. For patients with locally advanced, unresectable, and metastatic disease, platinum-based chemotherapy is routinely used; unfortunately, not all patients have a good response to the initial systemic therapy. Recently, the treatment options for bladder cancer are expanding rapidly. In patients at advanced stage, drugs targeting the checkpoints ‘programmed cell death 1’ (PD-1) and ‘programmed death ligand 1’ (PD-L1) were shown to induce rapid response [17,18]. Moreover, the combination of chemotherapy and immune therapy may result in synergistic efficacy through multiple mechanisms [19]. However, there is still no available targeted drug approved for the treatment of bladder cancer. Current research is exploring effective biomarkers and promising drug targets, along with the identification of novel molecular markers, as stated in the current EAU Guidelines of NMIBC, which state: “More
work is required to determine the role of molecular markers in improving the predictive accuracy of the currently existing risk tables” [20].

The TRIP13 gene has been shown to interact with a variety of proteins such as thyroid hormone receptors. As a modulator of chromosome recombination, TRIP13 was shown to participate in many processes of tumor progression. The most well-characterized protein interacting with TRIP13 is MAD2, which is a component of the composition of mitotic checkpoint complex, and is used in therapy influencing cell mitosis and proliferation. In the present study, we observed that TRIP13 knockdown significantly decreased the proliferation of bladder cancer cells, which reveals the oncogenic function of TRIP13 in bladder cancer and is consistent with the well-recognized role of TRIP13 in mitotic checkpoint complex constitution.

The correlation between TRIP13, mitosis, and proliferation has been reported many times; however, the relationship between TRIP13 and invasion had seldom been investigated, and the underlying mechanism has been unclear. The present study is the first to demonstrate that TRIP13 promotes proliferation and invasion of bladder cancer, and we further proved that TRIP13 is required in the EMT of bladder cancer, which may be a main cause of TRIP13-induced invasion. Previous evidence proved that TRIP13 could regulate G2-M transition and EMT by interacting with 14-3-3 protein zeta/delta protein (also known as YWHAZ) in colorectal cancer, or could promote metastasis of hepatocellular carcinoma by activating of TGF-β1/smad3 [10,11].

In the present study, we also proved that TRIP13 is required in the EMT and invasion of bladder cancer. Although the underlying mechanism and key effector downstream of TRIP13 is still unknown, our study provides insight into the clinical significance of TRIP13 and may stimulate interest in research on its mechanism study.

Figure 3. TRIP13 is required in the proliferation, invasion, and EMT of TCCSUP cells. (A) TRIP13 expression was silenced by siRNA in TCCSUP cells. (B) TRIP13 knockdown significantly impaired the proliferation of TCCSUP cells after 36-h culture. (C) Invasion ability of TCCSUP cells was remarkably decreased after knockdown of TRIP13. (D) The expression of E-cadherin was increased, while the expressions of N-cadherin and Snail were decreased after knockdown of TRIP13. In B and C, * and ** mean $P<0.05$ and $P<0.01$, respectively.
Conclusions

We, for the first time, demonstrated that TRIP13 is a prognostic biomarker in bladder cancer, and further showed that TRIP13 promotes the proliferation, invasion, and EMT of bladder cancer cells, indicating that TRIP13 may be a promising drug target for bladder cancer.

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Conflicts of interest

None.