Life Sciences 235 (2019) 116832

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

TIS111D can affect bladder cancer cells by regulating epithelial-mesenchymal transition

Yin Lei, Liu Yang, Jing Hongwei, Yu Hongyuan, Liu Tao*

Urology Surgery, First Affiliated Hospital of China Medical University, Shenyang, PR China

ARTICLE INFO

Keywords:
TIS11D
Bladder cancer
Epithelial-mesenchymal transition

ABSTRACT

Aims: Delineates the role of TIS111D in bladder cancer.

Materials and methods: The expression of TIS111D in bladder cancer and adjacent tissues was assessed by immunohistochemistry, Western blot and real-time PCR. Western blot and real-time PCR were used to analyse the expression of TIS111D in HT1197, T24, 5637 and TCCSUP cells. After TIS111D was silenced in T24, 5637 and TCCSUP cells, MTT and Transwell assays were used to detect the effects of TIS111D on proliferation and migration. Western blot and real-time PCR were used to detect the regulatory effect of downregulation of TIS111D on N-cad and E-cad. In vivo experiments confirmed the role of TIS111D in the growth and migration of bladder cancer and determined whether the role of TIS111D in bladder cancer is related to its regulation of N-cad and E-cad.

Key findings: The expression of TIS11D was higher in tumour tissues and bladder cancer cells. Si-TIS111D could inhibit the growth and migration of bladder cancer cells, while TIS111D could regulate the expression of E-cad and N-cad to regulate epithelial-mesenchymal transition (EMT). We also demonstrated that TIS111D could promote the growth and migration of bladder cancer in vivo by regulating EMT.

Significance: TIS111D may participate in the regulation of bladder cancer progression by regulating EMT.

1. Introduction

Bladder cancer has become the most common malignant tumour in the urinary system [1]. The occurrence of bladder cancer has attracted increasing attention, and related research has become an important topic in urology [2].

Metastasis of malignant tumours is a complicated pathological process, including many links and steps. In tumour cells with epithelial cell characteristics, the expression of proteolytic enzymes increases and their movement ability increases significantly. These changes can help tumour cells to metastasize and produce secondary tumours in distant locations. Epithelial cells can transform into mesenchymal cells only under specific physiological and pathological conditions, which is called epithelial-mesenchymal transformation (EMT) [3,4]. In the process of cell EMT, the expression of many kinds of proteins changes, among which, the decrease or loss of E-cadherin (E-cad) and the increase of N-cadherin (N-cad) are the most important markers of changes of EMT [5].

TIS11D, also known as BRF2, belongs to the zinc finger protein family. It can bind to AU-rich elements through its zinc finger structure to degrade mRNA or inhibit translation, and it plays a role in influencing inflammation and tumour occurrence [6]. In screening P53 target genes, the researchers found that TIS11D and p53 could interact in prostate cancer cells [7]. Other researchers found that TIS11D had experienced a frameshift mutation in cells from leukaemia and lymphoma patients [7]. TIS11D can affect the proliferation of HeLa cells and activate the S-phase cell cycle checkpoint [8–10]. However, there are few reports about TIS11D in bladder cancer. We examined whether the expression of TIS11D in bladder cancer is correlated with clinical characteristics, and what precisely is the function of TIS11D in bladder cancer.

In this study, we detected the function of TIS11D in bladder cancer with the ultimate aim of identifying potential targets for bladder cancer treatment.

2. Materials and methods

2.1. Ethics statement

The protocols of the clinic and laboratory were approved by the

* Corresponding author at: Urology Surgery, First Affiliated Hospital of China Medical University, No. 155, Nanjing Street, Heping District, Shenyang, Liaoning 110001, PR China.
E-mail address: jfwang@cmu.edu.cn (L. Tao).

https://doi.org/10.1016/j.lfs.2019.116832
Received 3 July 2019; Received in revised form 1 September 2019; Accepted 2 September 2019
Available online 03 September 2019
0024-3205/

Institute Research Ethics at the First Affiliated Hospital of China Medical University, China. All experiments were performed in accordance with the Declaration of Helsinki. All participating patients signed consent forms prior to enrolment in this study.

2.2. Patients and tissues

The cancer samples were obtained from 52 patients who had primary bladder cancers had undergone previous treatment before surgery and then underwent resection of bladder cancer from June 2011 to June 2012 at the Department of Urology, First Affiliated Hospital of China Medical University, Shenyang, China. All samples were immediately frozen in liquid nitrogen and stored at −80 °C. All samples were diagnosed according to the TNM stage by two senior pathologists independently.

2.3. Cell culture

Bladder cancer carcinoma cell lines TCCSUP, 5637 and T24, and HT-1197 (a noncarcinoma urinary bladder cell line) were purchased from the Cell Bank of the Typical Culture Preservation Committee of Chinese Academy of Sciences (Shanghai, China). All cells were cultured in 1640 medium (Invitrogen, Carlsbad, California, USA) supplemented with 10% foetal bovine serum (FBS; Invitrogen, Carlsbad, California, USA) with 95% O_2 and 5% CO_2 at 37 °C.

2.4. Immunofluorescence staining

The tissues were embedded in paraffin, fixed in formalin, and cut into 5 μm sections. They were dewaxed in xylene, rehydrated with graded ethanol, underwent quenching of endogenous peroxidase activity in 0.3% hydrogen peroxide, and antigen retrieval was conducted with microwave heating in a citric acid buffer solution. The samples were incubated overnight with anti-TIS11D antibody (sc-365908, 1:100, Santa Cruz Biotechnology, California, USA) at 4 °C. A DAB kit (Solarbio, Beijing, China) was used for colouring the bound antibodies. Subsequently, the sections were re-stained with haematoxylin (Beyotime Biotechnology, Shanghai, China). The slides were cleared with xylene and sealed with paraffin. The immunohistochemical results were judged by HSCORE (histological score) [11,12].

2.5. Real-time PCR

Total RNA was extracted by TRIzol reagent (Invitrogen, Carlsbad, California, USA) according to the manufacturer's protocol for tissues or cells. Real-time PCR was conducted using a SYBR green assay (Beyotime Biotechnology, Shanghai, China) with an Mx 3000P real-time PCR system (Applied Biosystems). All of the reactions were repeated at least three times. Gene expression levels were evaluated using the comparative Ct method. GAPDH was used as an internal control. Primer sequences are shown in Table 1.

2.6. Western blot analyses

Tissue and cells were lysed by RIPA (radio immunoprecipitation assay, Beyotime Biotechnology, Shanghai, China). Then, 30 μg protein from each sample was separated via 10% SDS-PAGE and transferred to a PVDF membrane. After blocking with 5% fat-free milk in TBST (Tris-buffered saline containing 0.1% Tween-20) for 2 h at room temperature, the membranes were then incubated overnight at 4 °C with specific antibodies against: TIS11D (sc-365908), E-cad (sc-8426), N-cad (sc-59987) and GAPDH (sc-365062) (Santa Cruz Biotechnology, California, USA) followed by anti-rabbit/mouse horseradish peroxidase conjugated IgG, and an ECL kit (Beyotime Biotechnology, Shanghai, China) was used for detection.

Table 1
Primers used in real-time PCR.

Name	Forward primer (5′– > 3′)	Reverse primer (5′– > 3′)
TIS11D	GGCCTCCAA AAGCGTT	AGCTGGCTCTGCGAATAGT
E-cad	CCCAGAGACTGGTGCCATTT	TCTGTGGCGATGATGAGAGC
N-cad	CGGTGCCATCATTGCCATCCT	GTCATAGTCCTGGTCTTCTTCTCC
GAPDH	GGTGCT-GAGTATGTCGTGGAGT	CAGTCTTCTGAGTGGCAGTGAT

2.7. RNAi knockdown

Cells were transfected with TIS11D siRNA (Qiagen, Valencia, CA) or negative control (NC) using Lipofectamine 2000 (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. The sequences were as follows: BRF2 siRNA: sense 5-GCACUUACAUGCAGA UAGUTT-3; antisense 5-ACUAUCUGCAUGUAAGUGCTT-3; NC: sense 5-UUCUCCGAACGUGUCACGUTT-3; antisense 5-ACGUGACACGUUCGG AGAATT-3. All experiments began 48 h after transfection.

2.8. MTT assay

A total of 1×10^3 cells were plated per cell in complete medium into 96-well plates. After transfection for 48 h, cells were cultured for 12, 24, 36 or 48 h. Then, 5 mg/ml of MTT (Beyotime Biotechnology, Shanghai, China) was added into each well. After 4 h, the medium was replaced by 0.1 ml DMSO. The results were measured as the optical density value at 490 nm by a Microplate Reader (BIO-RAD) [13].

2.9. Colony formation assay

After 48 h of transfection, the cells were digested and resuspended in a single-cell solution. The cells were inoculated into 6-well plates at a concentration of 1000 cells per well. After 2 w of culture, the culture was terminated when a clone became visible. They were fixed with methanol for 15 min and stained with Giemsa (Beyotime Biotechnology, Shanghai, China) for 15 min. Microscopic (Olympus 600 autobiochemical analyser) observation was used to analyse the clone formation rate. Colony-forming efficiency = colonies / plated cells × 100%.

2.10. Transwell assay

After 48 h of transfection, 1×10^4 cells in serum-free media were seeded in Transwell chambers (24-well plates, 8-μm pore size, Corning), while media containing 20% FBS was placed in the lower well. After incubation for 24 h, the non-invading cells were removed. The invading cells were stained with 0.1% crystal violet (Beyotime Biotechnology, Shanghai, China) and were counted under a microscope (Olympus 600 autobiochemical analyser).

2.11. Tumour xenograft implantation in nude mice

Four-week-old female athymic BALB/C nude mice were purchased from the Department of Animals, China Medical University (Shenyang, China). The TCCSUP cells were transfected with TIS11D siRNA or NC and then subcutaneously injected into nude mice. Tumour volumes were examined weekly and calculated according to the following: tumour volume (mm^3) = 0.5 × length × $width^2$. All nude mice were euthanized 6 weeks after injection and metastasis of the tumours was measured [14].

2.12. Statistical analysis

All data are presented as the mean ± SD from three independent experiments. Differences between groups were analysed by GraphPad

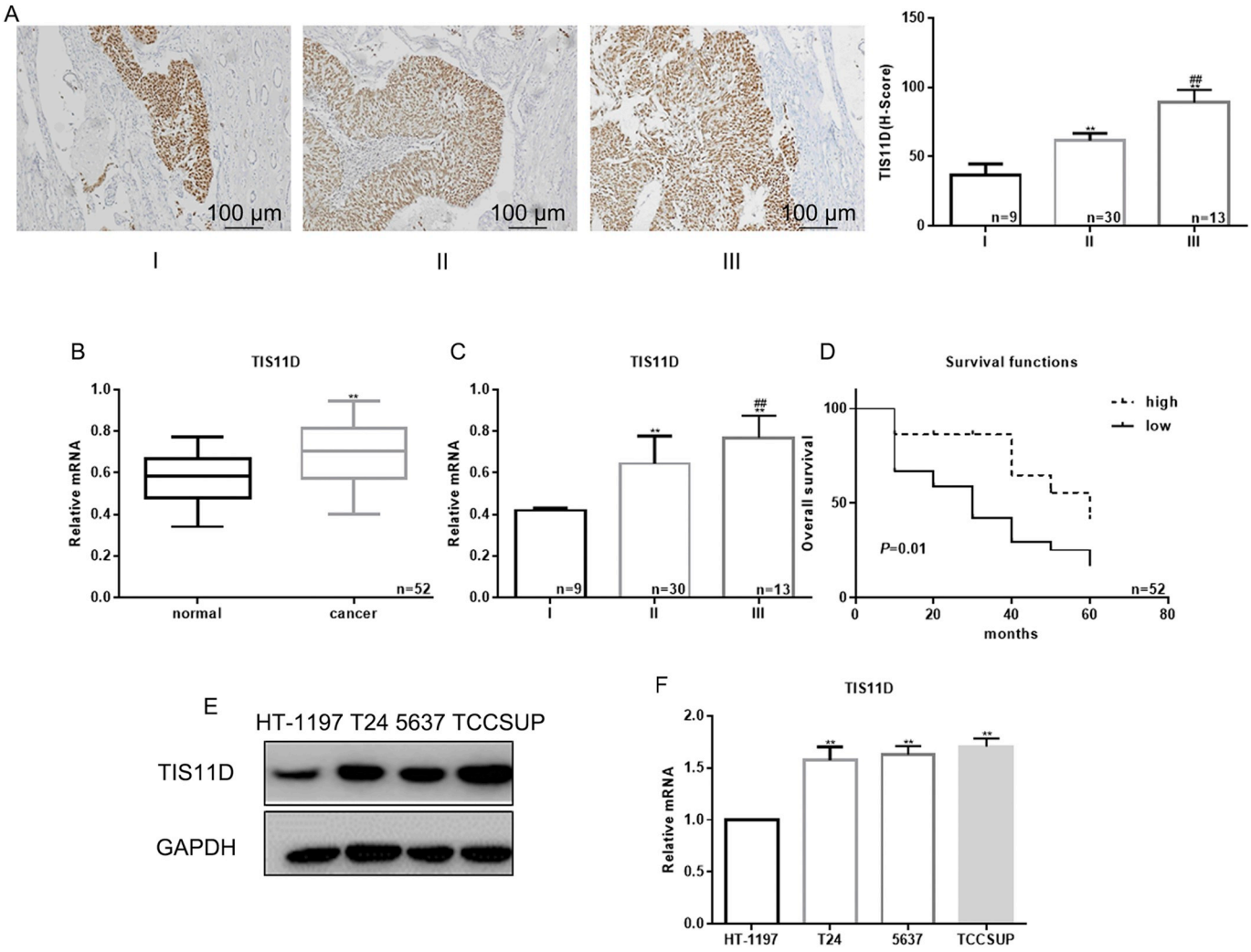


Fig. 1. TIS11D expression in bladder cancer. (A) The expression of TIS111D in bladder cancer tissues with different TNM grades was detected by immunohistochemical staining. $^{**}P < 0.05$ vs. I stage group, $^{\#\#}P < 0.05$ vs. II stage group. (B) TIS11D mRNA expression in 52 samples of bladder cancer tissues and normal tissues were detected respectively by real-time PCR. Data are shown as the mean ± SEM. $^{**}P < 0.05$ vs. normal group. (C) The expression of TIS111D in bladder cancer tissues with different TNM grades was detected by real-time PCR. Data are shown as the mean ± SEM. $^{**}P < 0.05$ vs. I stage group, $^{\#\#}P < 0.05$ vs. II stage group. (D) Patients' postoperative overall survival was recorded using the Kaplan-Meier model and compared between those with lower TIS11D expression and those with higher TIS11D expression ($P = 0.014$, log-rank test). (E) The protein level of TIS11D expression of different cell lines was detected with western blot. (F) The expression of TIS11D in different cell lines was detected by real-time PCR. Data are shown as the mean ± SEM. $^{**}P < 0.05$ vs. HT1197.

Table 2
The relationship between TIS11D and bladder cancer.

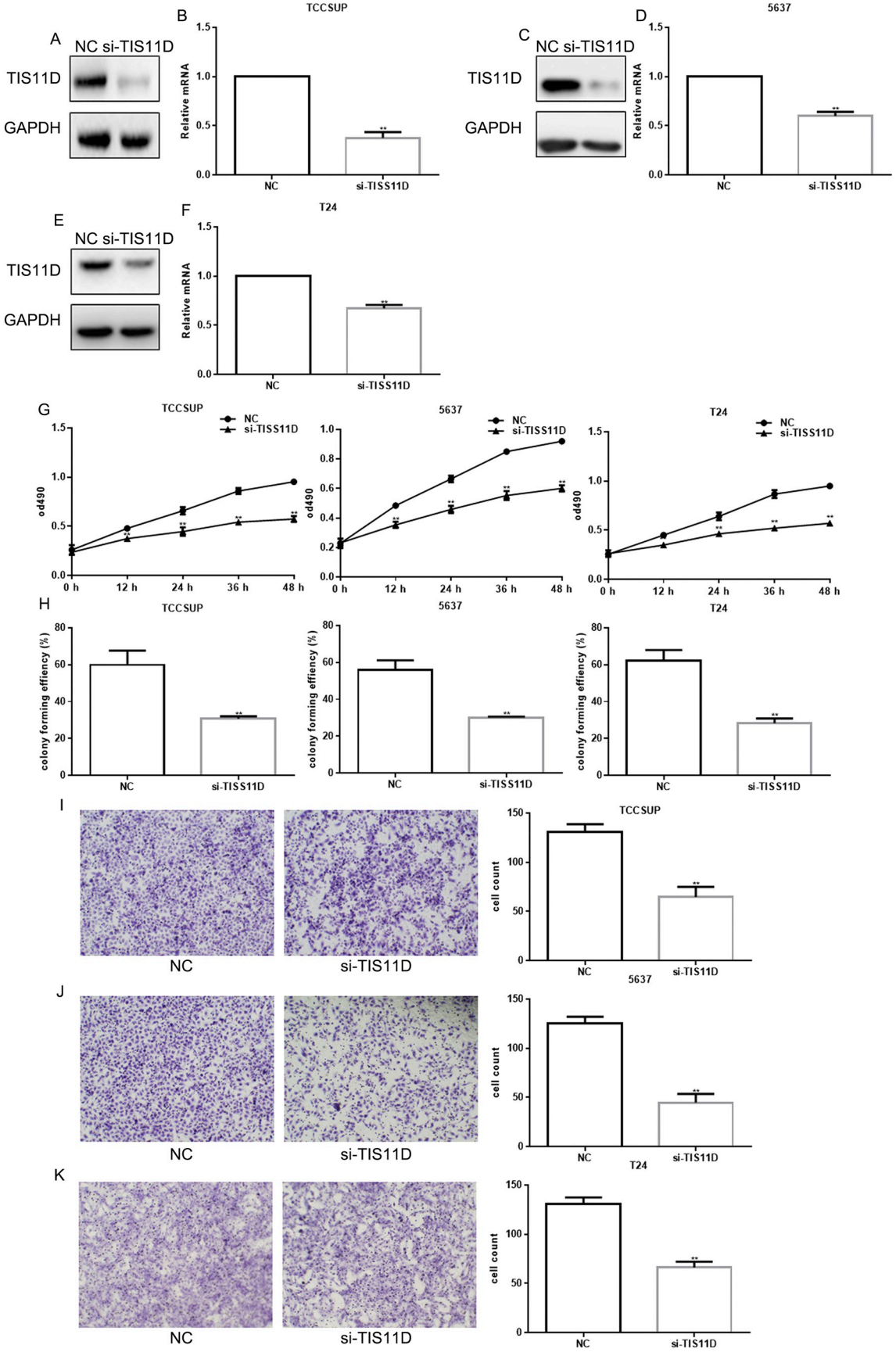
Clinicopathological factor		No.	Low expression	High expression	χ^2	P
Sex	Male	41	17	24	0.09	0.76
	Female	11	4	7		
Age	< 60	29	11	18	0.16	0.69
	≥60	23	10	13		
Differentiation	Well differentiated	21	15	6	9.60	0.01
	Moderate differentiated	19	8	11		
	Poorly differentiated	12	2	10		
Lymph node metastasis	With	13	2	11	4.50	0.03
	Without	39	19	20		
Clinical stage	I	9	7	2	8.60	0.01
	II	30	12	18		
	III	13	2	11		

Prism version 6.0 (GraphPad, USA) with the chi-square test, Student's *t*-test or one-way ANOVA. Overall survival was evaluated by Kaplan-Meier survival analysis with the log-rank test for comparison. $P < 0.05$ was considered statistically significant.

3. Results

3.1. TIS11D expression in bladder cancer

Immunohistochemical staining was used to detect the expression of TIS111D in bladder cancer tissues with different TNM grades. As shown in Fig. 1A, the expression of TIS111D increased with the grade of bladder cancer. The results of real-time PCR showed that TIS111D content in bladder cancer tissues was approximately 1.2 times higher than that in adjacent tissues and the expression of TIS111D increased with the grade of bladder cancer (Fig. 1B, C). In addition, Kaplan-Meier analysis revealed that a high expression level of TIS111D was significantly correlated with a shorter overall survival (Fig. 1D). The expression of TIS11D in TCCSUP, 5637 and T24 and HT-1197 cells was



(caption on next page)

Fig. 2. TIS11D promoted the proliferation and migration of bladder cancer cells. (A–F) The expression of TIS11D in different cell lines was detected by western blot and real-time PCR $^{**}P < 0.05$ vs. NC group. (G) Cells were transfected with si-TIS11D or NC, cell growth was detected by MTT assay. Data are shown as the mean ± SEM $^{**}P < 0.05$ vs. NC group. (H) Colony-forming efficiency of bladder cancer cells after transfection with si-TIS11D or NC. Data are shown as the mean ± SEM $^{**}P < 0.05$ vs. NC group. (I–K) Cells were transfected with si-TIS11D or NC, cell migration was detected by Transwell assay. Data are shown as the mean ± SEM $^{**}P < 0.05$ vs. NC group.

detected by western blot and real-time PCR (Fig. 1E, F). The results indicated that the expression of TIS111D in bladder cancer cells was higher than in normal cells.

The results also showed that the expression of TIS11D was correlated with bladder cancer prognosis (Table 2).

3.2. TIS11D promoted the proliferation and migration of bladder cancer cells

To detect the effect of TIS111D on the biological function of bladder cancer cells, we silenced the expression of TIS111D in different bladder cancer cell lines (Fig. 2A–F). MTT assay and colony formation assay showed that the growth of different bladder cancer cells was inhibited after expression of TIS111D was suppressed (Fig. 2G, H). Transwell assays showed that si-TIS11D could inhibit the migration of cells (Fig. 2I–K).

3.3. TIS11D influenced the expression of EMT-related proteins

As we all known, E-cad and N-cad are important proteins in the procession of EMT. Because TIS111D is highly expressed in bladder cancer tissues with strong invasive and metastatic abilities, and TIS111D has been proven to promote EMT [15], we examined the effect of si-TIS111D on E-cad and N-cad expression. Western blot and real-time PCR results showed that there was a significant difference between the NC and si-TIS11D groups in the expression of E-cad and N-cad (Fig. 3A–F).

3.4. si-TIS11D has a tumour-suppressing effect on in vivo bladder cancer development

To further evaluate the functional role of TIS11D in bladder cancer in vivo, si-TIS11D or NC stably transfected TCCSUP cells were injected into nude mice. si-TIS11D could significantly decrease the volume and metastasis of bladder cancer compared with the NC group (Fig. 4A–C). With the silencing of TIS11D, E-cad expression significantly increased and N-cad expression significantly decreased (Fig. 4D, E).

4. Discussion

Conventional radiotherapy, chemotherapy, hormone and immunotherapy are all very limited in the treatment of bladder cancer [16]. We hope that through our research we can identify targets for early diagnosis and advanced treatment of bladder cancer.

Previously, we detected TIS111D expression by immunohistochemistry and real-time PCR and found that TIS111D expression increased with the grade of bladder cancer. Real-time PCR also demonstrated that TIS111D was highly expressed in bladder cancer

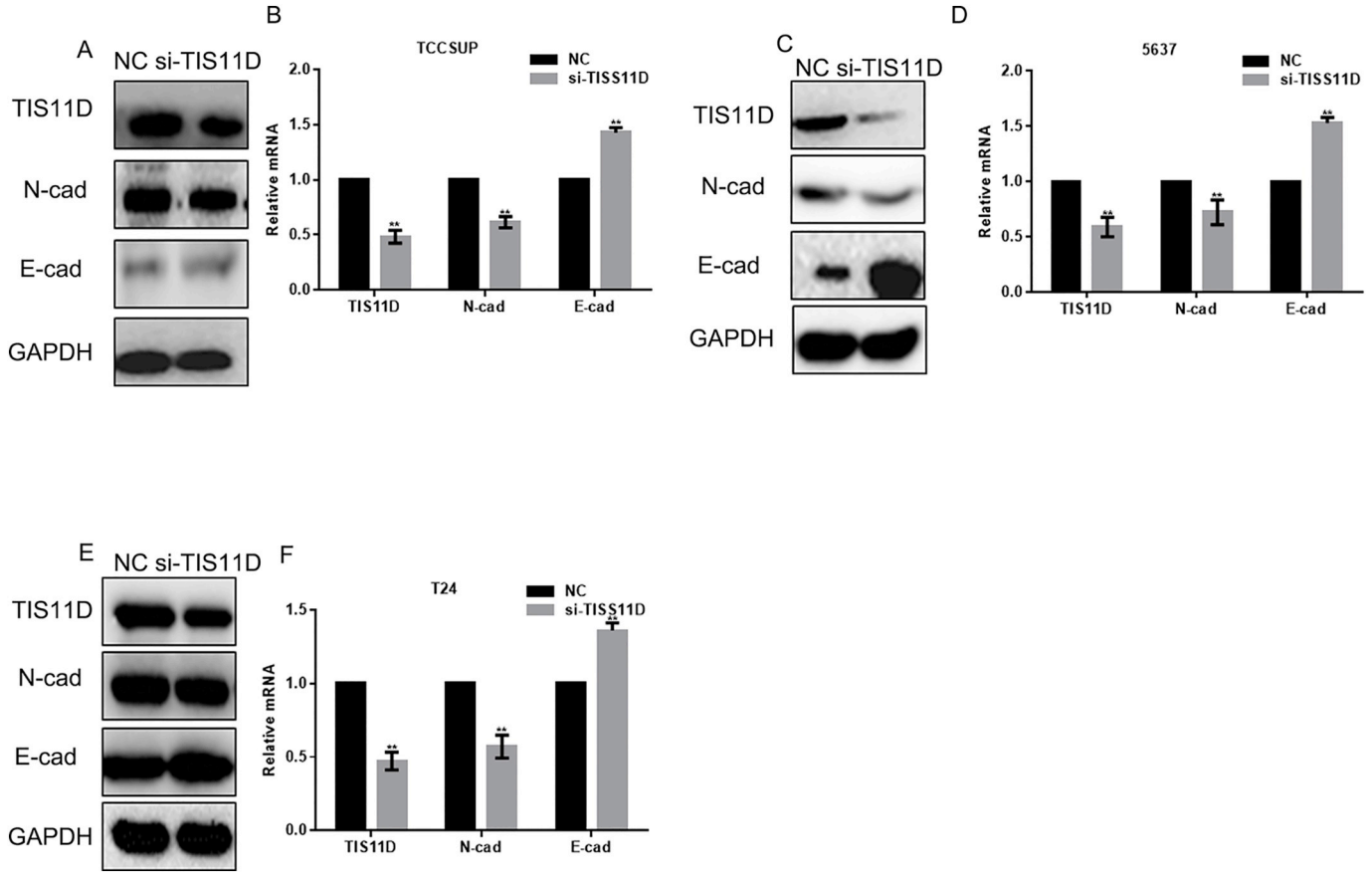


Fig. 3. TIS11D influenced the expression of EMT-related proteins. (A–F) Cells were transfected with si-TIS11D or NC, the expression of TIS11D, E-cad and N-cad were detected by western blot and real-time PCR $^{**}P < 0.05$ vs. NC group.

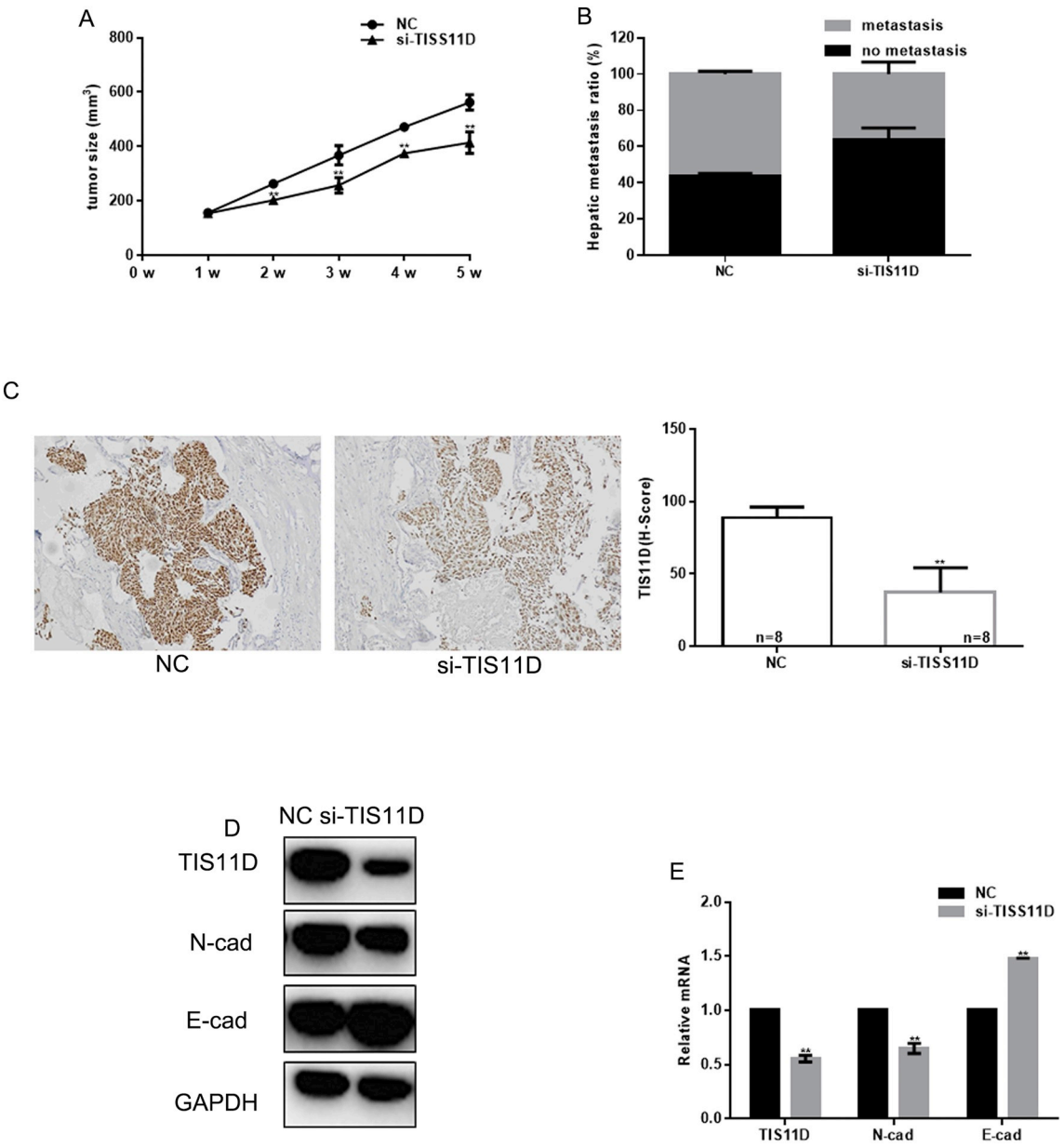


Fig. 4. si-TIS11D has a tumour-suppressing effect on in vivo bladder cancer development. (A) Tumour transplantations were evaluated weekly by calculating the in vivo volumes for 5 consecutive weeks. The results represent the mean ± SD of three experiments. $^{**}P < 0.05$ vs. NC group. (B) The tumour-bearing mice were killed after six weeks. The number of metastatic tumours was counted. The results represent the mean ± SD of three experiments. $^{**}P < 0.05$ vs. NC group. (C) Immunohistochemical staining was used to detect the expression of TIS11D in si-TIS11D and NC groups. $^{**}P < 0.05$ vs. NC group. (D, E) The expression of TIS11D, E-cad and N-cad were detected by western blot and real-time PCR $^{**}P < 0.05$ vs. NC group.

tissues relative to control tissues, and its expression was closely related to the survival time of patients with bladder cancer. These results suggest that the *TIS111D* gene may play an important role in the growth, invasion and metastasis of bladder cancer.

To further verify the role of TIS111D in bladder cancer cells, we measured the expression of TIS111D in TCCSUP, 5637, T24 and HT-1197 cells by western blot and real-time PCR. The results indicated that TIS111D was highly expressed in bladder cancer cells. After stable transfection of si-TISS11D, we found that si-TIS111D could significantly inhibit the growth and migration of bladder cancer cells.

In recent years, a large number of studies and increasing evidence have indicated that the mechanism of EMT is closely related to the invasive and metastatic abilities of malignant tumour cells [17]. Through the transformation between epithelial and mesenchymal phenotypes, tumour cells obtain a stronger migration ability and can more easily invade the extracellular matrix and pass through the vessel walls [18]. Therefore, the EMT phenomenon plays an important role in local invasion and distant organ metastasis of malignant tumours [19]. Through our experiments, we found that si-TIS11D can significantly promote the expression of E-cad and downregulate the expression of N-cad. This finding suggests that si-TIS111D may regulate the biological function of bladder cancer by promoting EMT. In in vivo experiments, we demonstrated that si-TIS111D can inhibit the growth and invasion of bladder cancer cells, and that this effect may be achieved by regulating the expression of E-cad and N-cad.

There are still some deficiencies in our study. We need more cases to confirm the role of TIS111D in bladder cancer. However, we still propose, for the first time, that TIS111D may be a potential therapeutic target in bladder cancer. TIS111D may participate in the regulation of bladder cancer progression by regulating EMT.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consent was obtained from all individual participants included in the study.

Declaration of competing interest

The authors declare no conflicts of interest.

References

[1] S.S. Islam, R.B. Mokhtari, A.S. Noman, M. Uddin, M.Z. Rahman, M.A. Azadi, A. Zlotta, T. van der Kwast, H. Yeger, W.A. Farhat, Sonic hedgehog (Shh) signaling promotes tumorigenicity and stemness via activation of epithelial-to-mesenchymal transition (EMT) in bladder cancer, Mol. Carcinog. 55 (5) (2016) 537–551.

[2] S.V. Williams, F.M. Platt, C.D. Hurst, J.S. Aveyard, C.F. Taylor, J.C. Pole, M.J. Garcia, M.A. Knowles, High-resolution analysis of genomic alteration on chromosome arm 8p in urothelial carcinoma, Genes Chromosom. Cancer 49 (7) (2010) 642–659.

[3] J. Luo, J. Chen, H. Li, Y. Yang, H. Yun, S. Yang, X. Mao, LncRNA UCA1 promotes the invasion and EMT of bladder cancer cells by regulating the miR-143/HMGB1 pathway, Oncol. Lett. 14 (5) (2017) 5556–5562.

[4] D.J. McConkey, W. Choi, L. Marquis, F. Martin, M.B. Williams, J. Shah, R. Svatek, A. Das, L. Adam, A. Kamat, A. Siefker-Radtke, C. Dinney, Role of epithelial-to-mesenchymal transition (EMT) in drug sensitivity and metastasis in bladder cancer, Cancer Metastasis Rev. 28 (3–4) (2009) 335–344.

[5] X. Pang, X. Fu, S. Chen, X. Zhu, H. Qi, Y. Li, F. Li, W. Tan, Overexpression of CIP2A promotes bladder cancer progression by regulating EMT, Clin. Transl. Oncol. 18 (3) (2016) 289–295.

[6] E. Iwanaga, T. Nanri, H. Mitsuya, N. Asou, Mutation in the RNA binding protein TIS11D/ZFP36L2 is associated with the pathogenesis of acute leukemia, Int. J. Oncol. 38 (1) (2011) 25–31.

[7] R.S. Jackson 2nd, Y.J. Cho, P. Liang, TIS11D is a candidate pro-apoptotic p53 target gene, Cell Cycle 5 (24) (2006) 2889–2893.

[8] B.R. Morgan, L.M. Deveau, F. Massi, Probing the structural and dynamical effects of the charged residues of the TZF domain of TIS11d, Biophys. J. 108 (6) (2015) 1503–1515.

[9] B.R. Morgan, F. Massi, A computational study of RNA binding and specificity in the tandem zinc finger domain of TIS11d, Protein Sci. 19 (6) (2010) 1222–1234.

[10] F. Qin, Y. Chen, Y.X. Li, H.F. Chen, Induced fit for mRNA/TIS11d complex, J. Chem. Phys. 131 (11) (2009) 115103.

[11] K. Nakahara, K. Yamasaki, T. Nagai, M. Fujii, T. Akioka, H. Takamori, N. Terada, S. Mukai, Y. Sato, T. Kamoto, Expression of protease activating receptor-2 (PAR-2) is positively correlated with the recurrence of non-muscle invasive bladder cancer: an immunohistochemical analysis, Res. Rep. Urol. 11 (2019) 97–104.

[12] J.A. March-Villalba, D. Ramos-Soler, P. Soriano-Sarrio, D. Hervas-Marin, L. Martinez-Garcia, J.M. Martinez-Jabaloyas, Immunohistochemical expression of Ki-67, cyclin D1, p16INK4a, and survivin as a predictive tool for recurrence and progression-free survival in papillary urothelial bladder cancer pTa / pT1 G2 (WHO 1973), Urol. Oncol. 37 (2) (2019) 158–165.

[13] S. Yang, A.M. Evens, S. Prachand, A.T. Singh, S. Bhalla, K. David, L.I. Gordon, Mitochondrial-mediated apoptosis in lymphoma cells by the diterpenoid lactone andrographolide, the active component of Andrographis paniculata, Clin. Cancer Res. 16 (19) (2010) 4755–4768.

[14] Y. Hu, C. Deng, H. Zhang, J. Zhang, B. Peng, C. Hu, Long non-coding RNA XIST promotes cell growth and metastasis through regulating miR-139-5p mediated Wnt/beta-catenin signaling pathway in bladder cancer, Oncotarget 8 (55) (2017) 94554–94568.

[15] Y. Tian, M. Lu, W. Yue, L. Li, S. Li, C. Gao, L. Si, L. Qi, W. Hu, H. Tian, TFIIB-related factor 2 is associated with poor prognosis of nonsmall cell lung cancer patients through promoting tumor epithelial-mesenchymal transition, Biomed. Res. Int. 2014 (2014) 530786.

[16] F. Wan, C. Cheng, Z. Wang, X. Xiao, H. Zeng, S. Xing, X. Chen, J. Wang, S. Li, Y. Zhang, W. Xiang, Z. Zhu, C. Johnson, et al., SATB1 overexpression regulates the development and progression in bladder cancer through EMT, PLoS One 10 (2) (2015) e0117518.

[17] C. Wang, A. Li, S. Yang, R. Qiao, X. Zhu, J. Zhang, CXCL5 promotes mitomycin C resistance in non-muscle invasive bladder cancer by activating EMT and NF-kappaB pathway, Biochem. Biophys. Res. Commun. 498 (4) (2018) 862–868.

[18] X. Wang, Z. Liang, X. Xu, J. Li, Y. Zhu, S. Meng, S. Li, S. Wang, B. Xie, A. Ji, B. Liu, X. Zheng, L. Xie, miR-148a-3p represses proliferation and EMT by establishing regulatory circuits between ERBB3/AKT2/c-myc and DNMT1 in bladder cancer, Cell Death Dis. 7 (12) (2016) e2503.

[19] Q. Zhang, Z. Mao, J. Sun, NF-kappaB inhibitor, BAY11-7082, suppresses M2 tumor-associated macrophage induced EMT potential via miR-30a/NF-kappaB/snail signaling in bladder cancer cells, Gene 710 (2019) 91–97.