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LncRNA TMPO-AS1 promotes proliferation and migration in bladder cancer

Y.-C. HE, Y.-G. BI, L. JIANG

Department of Urology Surgery, Caoxian People's Hospital, Heze, China

Abstract. – OBJECTIVE: This study aims to uncover the *in vitro* influences of lncRNA TMPO-AS1 on the progression of bladder cancer (BLCA) and the underlying mechanism.

PATIENTS AND METHODS: Expression levels of TMPO-AS1 in BLCA tissues and normal bladder tissues were analyzed in The Cancer Genome Atlas (TCGA) database. Differential expressions of TMPO-AS1 in BLCA tissues and normal bladder epithelial tissues were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Potential influence of TMPO-AS1 on prognosis of BLCA patients was assessed. *In vitro* influences of TMPO-AS1 on proliferative and migratory abilities in T24 and UMUC-3 cells were evaluated by Cell Counting Kit-8 (CCK-8), transwell, and wound healing assay, respectively. Finally, the correlation between TMPO-AS1 and its sense RNA TMPO was assessed by analyzing TCGA database, clinical samples, and BLCA cell lines.

RESULTS: By analyzing TCGA database and clinical samples, it was found that TMPO-AS1 was upregulated in BLCA tissues compared with that in normal bladder tissues. Worse survival was observed in BLCA patients with high expression of TMPO-AS1. TMPO-AS1 level was correlated to muscle invasiveness and TNM stage of BLCA patients. In T24 and UMUC-3 cells, the knockdown of TMPO-AS1 suppressed proliferative and migratory abilities. TMPO-AS1 level was positively correlated to that of its sense RNA TMPO. Moreover, the knockdown of TMPO-AS1 downregulated mRNA and protein levels of TMPO in BLCA cells.

CONCLUSIONS: TMPO-AS1 is upregulated in BLCA tissue and closely linked to poor prognosis of BLCA patients.

Key Words:
BLCA, TMPO-AS1, Proliferation, Migration, Survival analysis.

Introduction

Bladder cancer (BLCA) is a common malignancy in the urogenital system with high incidence and mortality[1,2]. The overall prognosis and 5-year survival of BLCA are unsatisfactory even after active treatment[3]. However, the effective and sensitive hallmarks for predicting the prognosis and monitoring disease progression are lacking[4,5]. Thus, it is necessary to clarify the molecular mechanism of BLCA, so as to improve the therapeutic efficacy in BLCA patients.

Long non-coding RNAs (lncRNAs) are non-coding RNAs without biological functions of protein translation[6]. So far, lncRNAs have been discovered to exert a carcinogenic or anti-tumor effect[7]. With the rapid improvement on the next-generation sequencing technology, abundant abnormally expressed lncRNAs have been identified to affect every aspect of tumor progression, including tumor cell proliferation, drug resistance, and tumor metastasis[8]. It is suggested that lncRNAs could regulate the expression levels of miRNAs, mRNAs, and proteins[9]. In particular, regulatory effects of sense RNAs on lncRNAs are well concerned. SPINT1-AS1 and its sense RNA SPIMT1 serve as diagnostic hallmarks in esophageal carcinoma[10]. LncRNA OIP5-AS1 deficiency affects expression and transcription of its downstream gene OIP5, thus regulating proliferative and apoptotic capacities in BLCA[11]. In breast cancer, antisense RNAs affect mRNA transcription by mediating the stability of its sense RNAs, thereafter regulating tumor progression[12].

TMPO-AS1 is a natural antisense RNA, which plays an oncogenic role in non-small cell lung cancer. It is reported[13] that TMPO-AS1 results in poor prognosis of lung cancer by aggravating the malignant phenotypes *via* mediating translational level of its sense RNA TMPO. Our previous study uncovered dysregulation of TMPO-AS1 in BLCA. In this paper, we mainly explored potential influences of TMPO-AS1 on the progression of BLCA and the involvement of its sense RNA TMPO.

Corresponding Author: Lin Jiang, BM; e-mail: heyongchao2005@163.com

Patients and Methods

Data Analysis on GEPIA

Differential expressions of TMPO-AS1 in BLCA tissues and normal bladder tissues were analyzed based on the visualized website of The Cancer Genome Atlas (TCGA), GEPIA[14]. A relevant dataset containing 404 BLCA tissues and 28 normal bladder tissues was downloaded for analyzing TMPO-AS1 level and potential correlation between the expression levels of TMPO-AS1 and TMPO.

Sample Collection

Forty BLCA tissues and adjacent bladder epithelial tissues were collected from Caoxian People's Hospital, which were stored at -80°C after pathological confirmation. None of BLCA patients received preoperative anti-tumor treatments. Their clinical data were listed in Table I. Patients and their families in this study have been fully informed. This study was approved by Ethics Committee of Caoxian People's Hospital and was conducted in accordance with the Declaration of Helsinki.

Cell Culture

BLCA cell lines (T24, RT4, J82, and UMUC-3) and normal bladder epithelial cell line (SV-HUC-1) were provided by Cell Bank, Chinese Institute of Science (Shanghai, China). T24, RT4, and J82 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA), UMUC-3 cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA), and SV-HUC-1 cells were cultured in F-12. Culture medium was supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 μg/mL streptomycin and 100 IU/mL penicillin. They were maintained in an incubator with 37°C, 5% CO_2. Culture medium was regularly replaced.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Cellular RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA), quantified using a spectrometer, and reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using the PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). SYBR Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan) was utilized for qRT-PCR. Relative level was calculated using $2^{-\Delta\Delta Ct}$ method. The primer sequences are listed as follows: TMPO-AS1: 5'-AGCCAGACCTCTACAATCGG-3' (forward) and 5'-TTAGGATTCTTGCGGGTGGT-3' (reverse); TMPO: 5'-CCCCTCGGTCCTGACAAAAG-3' (forward) and 5'-CGCTCTTCGTCACTGGAGAA-3' (reverse); glyceraldehyde 3-phosphate dehydrogenase (GAPDH): 5'-CTGGGCTACACTGAGCACC-3' (forward) and 5'-AAGTGGTCGTTGAGGGCAATG-3' (reverse).

Transfection

TMPO-AS1 siRNA and si-NC were provided by GenePharma (Shanghai, China). The

Table I. Correlation between TMPO-AS1 expression and clinical parameters of bladder cancer patients.

Clinical parameters		N = 40	TMPO-AS1 Expression High	TMPO-AS1 Expression Low	*p*-value
Sex	Female	13	5	8	0.8904
	Male	27	11	16	
Age (year)	< 50	9	3	6	0.3337
	≥ 50	31	16	15	
Stage	I-II	28	11	17	0.0106*
	III-IV	12	10	2	
Tumor size (cm)	< 3	22	14	8	0.6038
	≥ 3	18	10	8	
Invasion (muscle)	Yes	16	11	5	0.0137*
	No	24	7	17	
Lymphatic metastasis	Yes	11	5	6	0.4548
	No	29	17	12	
Distant metastasis	Yes	7	5	2	0.1218
	No	33	13	20	
Histological grade	High	17	11	6	0.6015
	Low	23	13	10	

cells were transfected with 50 nM plasmid using HilyMax (Dojindo Molecular Technologies, Kumamoto, Japan). Sequences of siRNAs are listed as follows: si-TMPO-AS1: 5′-GAGCCGAACUACGAACCAATT-3′ and 5′-UUGGUUCGUAGUUCGGCUCTT-3′; si-NC: 5′-UUCUCCGAACGUGUCACGUTT-3′ and 5′-ACGUGACACGUUCGGAGAATT-3′.

Cell Counting Kit-8 (CCK-8) Assay

The cells were inoculated into 96-well plates with 1×10^3 cells per well. At the appointed time points, 10 μL of CCK-8 solution (Dojindo Molecular Technologies, Kumamoto, Japan) was added in each well. The absorbance at 450 nm of each sample was measured by a microplate reader (Bio-Rad, Hercules, CA, USA).

Transwell Assay

1×10^5 cells suspended in 100 μL of serum-free medium were inoculated on the upper chamber of a transwell insert (8 mm pore size; Corning, Corning, NY, USA). In the bottom side, 500 μL of medium containing 10% FBS was applied. After 48 h of incubation, the cells migrated to the bottom side were fixed in methanol for 15 min, dyed with crystal violet for 20 min, and counted using a microscope (Olympus, Tokyo, Japan).

Wound Healing Assay

The cells were inoculated in 6-well plates and cultured to 70% confluence. An artificial wound was created in the confluent cell monolayer using a 200 μL pipette tip. Wound closure was captured at 0 and 24 h, respectively.

Western Blot

Cellular protein was extracted, and its concentration was determined (Beyotime, Shanghai, China). Protein sample was loaded on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland), and blocked in 5% skim milk for 1 hour, followed by incubation with primary and secondary antibodies. After washing with 1×Tris Buffered Saline and Tween 20 (TBST) for 1 min, the chemiluminescent substrate kit was used for exposure of the protein band.

Statistical Analysis

GraphPad 7.0 (La Jolla, CA, USA) was used for data analysis. Data were expressed as mean±SD (standard deviation). Kaplan-Meier method was introduced for survival analysis. Data between the two groups were compared using the *t*-test. The correlation between TMPO-AS1 and clinical data of BLCA patients was analyzed by Chi-square test. $p<0.05$ considered the difference was statistically significant.

Results

Upregulation of TMPO-AS1 in BLCA

By analyzing the downloaded TCGA dataset, TMPO-AS1 was found to be upregulated in BLCA tissues compared with that of normal bladder tissues (Figure 1A). Similarly, TMPO-AS1 was highly expressed in BLCA tissues collected in our hospital compared to those of normal blad-

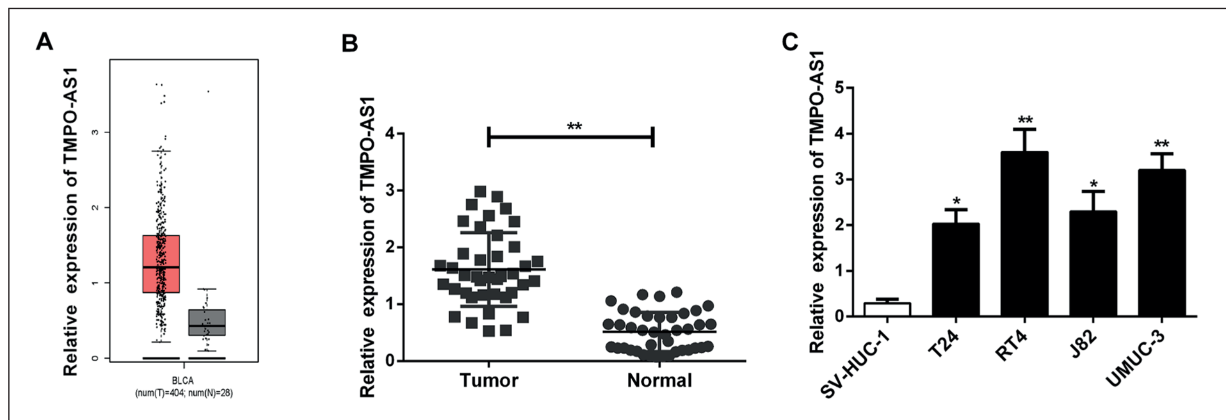

Figure 1. Upregulation of TMPO-AS1 in BLCA. **A,** TMPO-AS1 levels in BLCA tissues (n=404) and normal bladder tissues (n=28) analyzed in TCGA. **B,** TMPO-AS1 levels in collected BLCA tissues (n=40) and normal bladder epithelial tissues (n=40). **C,** TMPO-AS1 level in BLCA cell lines (T24, RT4, J82 and UMUC-3) and normal bladder epithelial cell line (SV-HUC-1). *$p<0.05$, **$p<0.01$.

der epithelial tissues (Figure 1B). *In vitro* abundance of TMPO-AS1 remained higher in BLCA cell lines as well (Figure 1C). It is suggested that TMPO-AS1 was involved in the progression of BLCA.

TMPO-AS1 Level was Correlated to Invasiveness, TNM Stage and Poor Prognosis in BLCA Patients

Clinical data of enrolled 40 BLCA cases were analyzed. Kaplan-Meier curves revealed that BLCA patients with high expression of TMPO-AS1 had worse prognosis (Figure 2). Chi-square test uncovered that TMPO-AS1 level was correlated to muscle invasiveness and TNM stage in BLCA patients (Table I). In BLCA patients with muscle invasiveness, the ratio of those expressing high level of TMPO-AS1 was 68.75%. Nevertheless, TMPO-AS1 level was unrelated to sex, age, tumor size, lymphatic metastasis, distant metastasis, and histological stage of BLCA patients.

Knockdown of TMPO-AS1 Suppressed Proliferative and Migratory Abilities in BLCA

To uncover biological function of TMPO-AS1 in BLCA cells, the transfection efficacy of si-TMPO-AS1 was first verified in T24 and UMUC-3 cells (Figure 3A). CCK-8 assay showed the decreased viability in BLCA cells transfected with si-TMPO-AS1 (Figure 3B). In addition, the number of migrating BLCA cells was reduced after the knockdown of TMPO-AS1 (Figure 3C). Wound healing assay further demonstrated that silencing of TMPO-AS1 reduced the percentage of wound closure, suggesting the attenuated migratory ability (Figure 3D). The above data demonstrated that TMPO-AS1 is able to promote the proliferative and migratory abilities in BLCA.

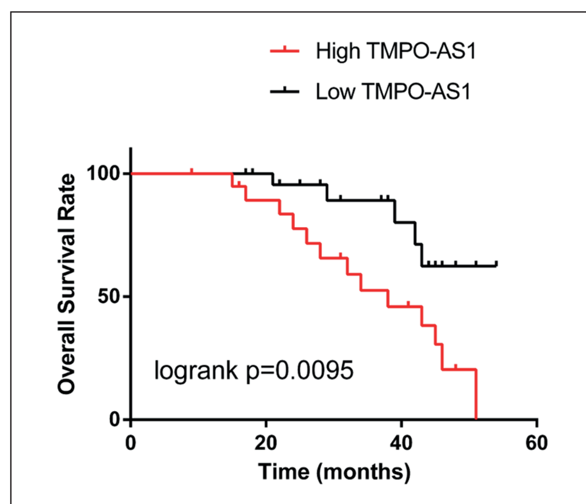

Figure 2. TMPO-AS1 level was correlated to poor prognosis in BLCA patients. Worse survival in BLCA patients expressing high level of TMPO-AS1 than those with low level. $^{**}p<0.01$.

Correlation Between TMPO-AS1 and TMPO in BLCA

A previous study has pointed out that TMPO-AS1 is able to change biological functions of tumor cells by altering mRNA level of its sense RNA TMPO. Here, we analyzed the potential correlation between the expression levels of TMPO-AS1 and TMPO in TCGA, and a positive correlation was identified (Figure 4A). A positive correlation between TMPO-AS1 and TMPO levels in collected BLCA tissues was identically obtained in Pearson correlation test (R^2=0.1773, Figure 4B). Interestingly, both mRNA and protein levels of TMPO in BLCA cells were downregulated after the knockdown of TMPO-AS1 (Figure 4C, 4D). It is suggested that TMPO-AS1 was capable of aggravating the malignant progression of BLCA by suppressing transcription and protein translation of its sense RNA TMPO.

Discussion

In recent years, lncRNAs have been a hot topic in tumor research. They are non-invasive diagnostic indicators that could be used in clinical application[15]. Natural antisense RNAs are critical regulators involved in biological processes. It is believed that antisense RNAs participate in tumor progression by mediating transcription activity, protein translation and RNA stability of the corresponding sense mRNAs[16]. TMPO-AS1 is a natural antisense RNA located on the anti-chain of TMPO, which is dysregulated in many types of tumors[17-19]. In lung cancer, TMPO-AS1 serves as a ceRNA sponging downstream miRNAs, which is considered as a prognostic indicator[17]. Later, TMPO-AS1 is discovered to exert a crucial function in lung cancer *via* affecting transcription of the corresponding sense mRNA.

In this paper, TCGA database and collected tissue samples in our hospital were analyzed, and upregulated TMPO-AS1 was found in BLCA tissues. High level of TMPO-AS1 was closely

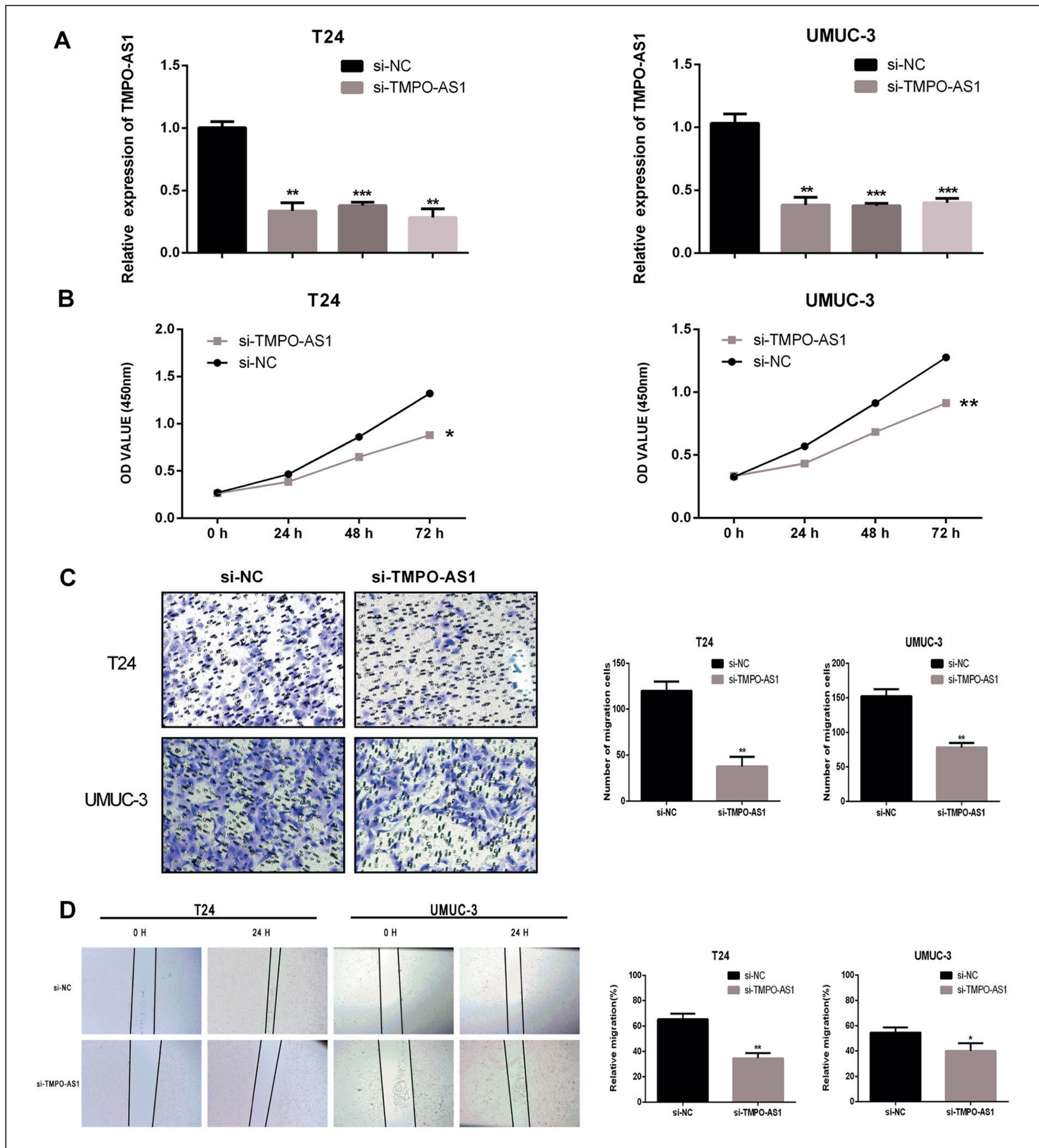

Figure 3. Knockdown of TMPO-AS1 suppressed proliferative and migratory abilities in BLCA. **A,** Transfection efficacy of si-TMPO-AS1 in T24 and UMUC-3 cells. **B,** Viability in T24 and UMUC-3 cells transfected with si-TMPO-AS1 or si-NC. **C,** Number of migratory cells in T24 and UMUC-3 cells transfected with si-TMPO-AS1 or si-NC (magnification: 40x). **D,** Percentage of wound closure in T24 and UMUC-3 cells transfected with si-TMPO-AS1 or si-NC (magnification: 40x). *$p<0.05$, **$p<0.01$, ***$p<0.001$.

correlated to muscle invasiveness, TNM stage, and poor prognosis of BLCA patients. *In vitro* experiments demonstrated the promotive effects of TMPO-AS1 on proliferative and migratory abilities in T24 and UMUC-3 cells.

As a potential target gene of TMPO-AS1, TMPO is a thymopoietin applied in the immune therapy of malignant tumors[20]. Our study revealed that dysregulated TMPO-AS1 was positively correlated to the expression level of its sense RNA

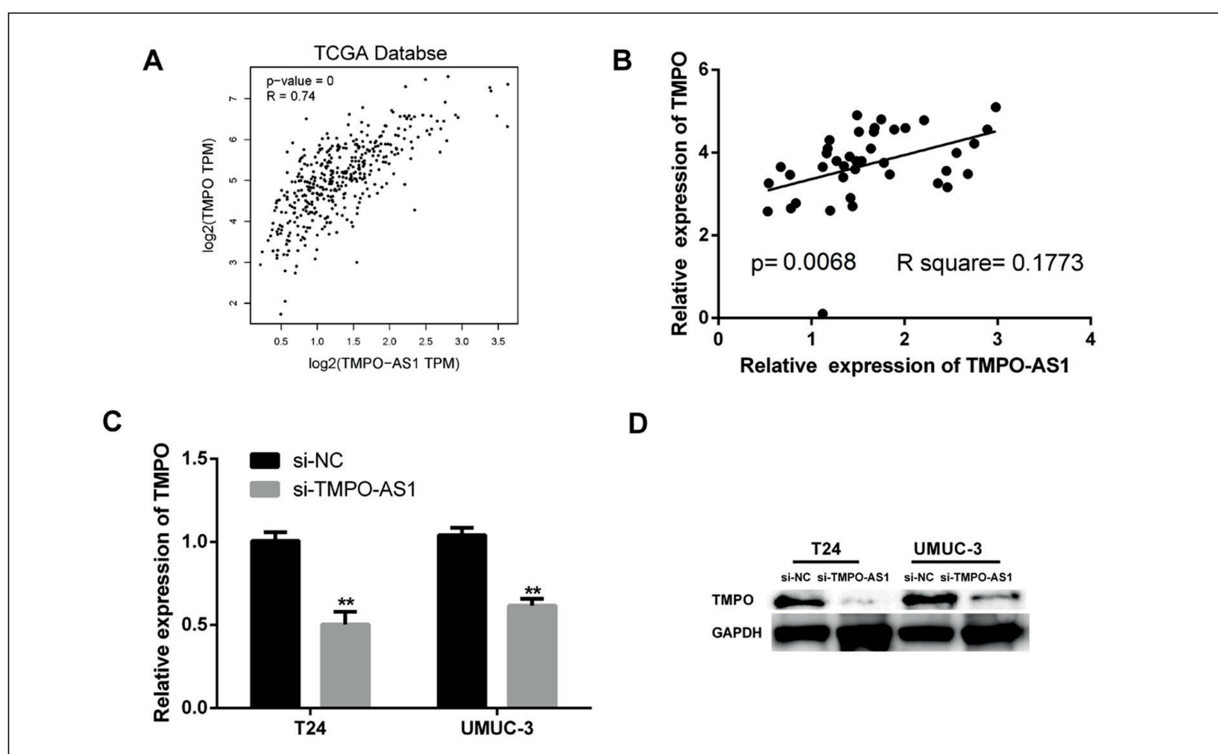

Figure 4. Correlation between TMPO-AS1 and TMPO in BLCA. **A,** A positive correlation between TMPO-AS1 and TMPO in BLCA tissues analyzed by TCGA database. **B,** A positive correlation between TMPO-AS1 and TMPO in collected BLCA tissues. **C, D,** The mRNA (**C**) and protein levels (**D**) of TMPO in T24 and UMUC-3 cells transfected with si-TMPO-AS1 or si-NC. ***$p<0.01$.

TMPO. The knockdown of TMPO-AS1 blocked transcription and protein translation of TMPO, thereafter aggravating the progression of BLCA. The regulatory relationship between TMPO-AS1 and TMPO contributes to be a new breakthrough in clinical treatment of BLCA.

Conclusions

This study demonstrated that TMPO-AS1 aggravates the malignant progression of BLCA by promoting proliferative and migratory abilities *via* interacting with its sense mRNA TMPO.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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