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XTP8 stimulates migration and invasion of gastric carcinoma through interacting with TGIF1

K. JIA[1], Q.-H. WEN[2], X. ZHAO[3], J.-M. CHENG[4], L. CHENG[5], M. XI[6]

[1]Department of General Surgery, Shanxi Cancer Hospital, Taiyuan, China
[2]Department of Clinical Laboratory, Dongguan People's Hospital, Dongguan, China
[3]Shanxi Province Centers for Disease Control and Prevention, Taiyuan, China
[4]Department of Pathology and Central Laboratory, Heze Medical College, Heze, China
[5]Department of Clinical Laboratory, Huadu District People's Hospital/Huadu Hospital Affiliated to Southern Medical University, Guangzhou, China
[6]Department of Urology, Huadu District People's Hospital/Huadu Hospital Affiliated to Southern Medical University, Guangzhou, China

Kai Jia and *Qinghui Wen* contributed equally to this work

Abstract. – OBJECTIVE: To determine expression characteristics of XTP8 and TGIF1 in gastric carcinoma (GC), and the potential roles of XTP8/TGIF1 axis in influencing the progression of GC.

MATERIALS AND METHODS: The expression levels of XTP8 and TGIF1 in GC tissues and cells were detected. Their functions in prognosis in GC patients were evaluated by the Kaplan-Meier method. The correlation between the XTP8 level and the pathological indexes of the GC patients were analyzed. The changes in the proliferation, migration, and invasion capacities of MKN-45 and SGC-7901 cells affected by XTP8 and TGIF1 were assessed. The interaction between XTP8 and TGIF1 was determined through Dual-Luciferase reporter gene assay and rescue experiments.

RESULTS: XTP8 was upregulated in GC tissues and cells. XTP8 level was positively correlated with lymphatic and distant metastasis, as well as poor prognosis of GC patients. The silence of XTP8 attenuated proliferation, migration, and invasion capacities of MKN-45 and SGC-7901 cells. TGIF1 was the downstream gene binding to XTP8, which was downregulated in GC, and XTP8 negatively regulated the TGIF1 level in GC tissues. Importantly, the knockdown of TGIF1 could abolish the regulatory effect of XTP8 on GC cell behaviors.

CONCLUSIONS: XTP8 is upregulated in GC and is closely linked to lymphatic metastasis, distant metastasis, and poor prognosis of GC patients. Besides, it accelerates the malignant progression via negatively regulating TGIF1.

Key Words:
XTP8, TGIF1, Gastric carcinoma, Metastasis.

Introduction

Gastric carcinoma (GC) is a prevalent malignancy in the world, and is the second leading cause of tumor deaths[1-3]. The majority of GC patients progress into the advanced stage at the initial diagnosis, accompanied with lymphatic and distant metastasis[3-5]. Due to great advance achieved in surgical technologies and adjuvant therapies, the mortality of GC sharply decreases. Nevertheless, the 5-year survival rate of advanced GC maintains at 30-40%[6-8]. It is urgent to clarify the etiology and pathogenesis of GC, and to develop the molecular hallmarks for early-stage diagnosis, individualized therapy, and prognosis assessment[9,10].

XTP8 protein (HBV X antigen activating protein 8) was first isolated in 2003, which is an HBx transactivation gene screened by suppression subtractive hybridization[11]. XTP8 locates on chromosome 5q12.1 and contains a 1590 bp CDS region encoding 529 amino acid residues, and it is also known as DEPDC1B or XTP1[11,12]. XTP8 is involved in the development of various types of cancers, including hepatocellular carcinoma[12]. The biological functions of XTP8 in GC remain unclear.

Strong invasiveness and metastasis are the major manifestations of GC, involving alterations in multiple genes and pathways[13-15]. TGIF1 belongs to the TALE superfamily and participates in

Corresponding Authors: Lu Cheng, MM; e-mail: lululu3683@sina.com
Ming Xi, MM; e-mail: bulesimon@sina.com

many biological processes, including embryonic development, cell growth, and differentiation[16,17]. TGIF1 can control the malignant progression by affecting the metastasis of tumor cells[17]. As a co-suppressor molecule, TGIF1 is able to block TGF-β signaling in different ways[18]. In this paper, the interaction between XTP8 and TGIF1, and their functions in influencing the progression of GC were mainly discussed.

Patients and Methods

Patients and GC Samples

A total of 37 pairs of GC tissues and adjacent normal tissues were harvested from GC patients undergoing surgery. Baseline characteristics and their follow-up data were recorded. Patients and their families have been fully informed. This investigation was approved by the Ethics Committee of the Shanxi Cancer Hospital. This study was conducted in accordance with the Declaration of Helsinki.

Cell Culture

A human gastric mucosal cell line GES-1 and GC cell lines AGS, BGC-823, MKN-45, and SGC-7901 were provided by American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and maintained in a 5% CO_2 incubator at 37°C. Cell passage was conducted at 80-90% confluence.

Transfection

The cells were inoculated in a 6-well plate and cultured to achieve 70% confluence. Transfection was conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) For 48 h, and then, the transfected cells were harvested for functional experiments. The transfection plasmids were constructed by GenePharma (Shanghai, China).

Cell Counting Kit (CCK-8) assay

The cells wells were inoculated in a 96-well plate at 2×10^3 cells per well. On day 1, 2, 3, and 4, the absorbance value at 450 nm of each sample was recorded using the CCK-8 kit (Dojindo Molecular Technologies, Kumamoto, Japan) for plotting the viability curves.

Transwell migration and invasion Assays

The cells were inoculated in a 24-well plate with 5.0×10^5/mL. Then, 200 μL of suspension was applied in the upper transwell chamber (Millipore, Billerica, MA, USA) and inserted in a 24-well plate, whereas 500 μL of medium containing 10% FBS was applied in the lower chamber. After 48 h of incubation, the cells penetrating to the lower chamber were fixed in methanol for 15 min, dyed with crystal violet for 20 min, and counted using a microscope. Finally, the penetrating cells were counted in 5 randomly selected fields per sample (20×).

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

The cells were lysed in TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Extracted RNAs were purified by DNase I treatment, and reversely transcribed into cDNA using PrimeScript RT Reagent (TaKaRa, Otsu, Shiga, Japan). After that, the obtained cDNA was subjected to qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan), with β-actin and U6 as internal references. Each sample was performed in triplicate, and the relative level was calculated by $2^{-\Delta\Delta Ct}$. The primer 5.0 was used for designing qRT-PCR primers, and the primer sequences are listed as follows: XTP8: forward: 5'-AGCTACCAGGCTGTGGAATG-3', reverse: 5'-AGCTCTTGAAACGACAGCGA-3', TGIF1: forward: 5'-ACCAATCACATAGCCCTGCC-3', reverse: 5'-TCAGAGCTGCAGATGTGGTC-3', GAPDH: forward: 5'-CGCTCTCTGCTCCTCCTGTTC-3', reverse: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Dual-Luciferase Reporter Gene Assay

Wild-type and mutant-type XTP8 Luciferase vectors were constructed based on the binding sites in the promoter regions of XTP8 and TGIF1. Then, the cells were co-transfected with pmirGLO-XTP8-WT/pmirGLO-XTP8-MUT/pmirGLO and NC/pcDNA-TGIF1. After co-transfection for 48 h, the cells were lysed for determining the Luciferase activity.

Statistical Analysis

Data were analyzed using GraphPad Prism 5 V5.01 (Version X; La Jolla, CA, USA) and expressed as mean ± standard deviation. The differences between the two groups were assessed by the *t*-test. The Kaplan-Meier curves were utilized for survival analysis, followed by the Log-rank test to compare the differences between the two curves. The Spearman correlation test was performed to

assess the relationship between the two genes. $p<0.05$ was considered as statistically significant.

Results

XTP8 Was Highly Expressed in GC

A total of 37 pairs of GC tissues and adjacent normal tissues were collected to determine the differential expression of XTP8. As qRT-PCR data revealed, XTP8 was highly expressed in GC tissues relative to normal ones (Figure 1A). Similarly, XTP8 was upregulated in GC cells than that of human gastric mucosal cells (Figure 1B).

XTP8 Level Was Correlated With Lymphatic Metastasis, Distant Metastasis, and Survival of GC

The baseline characteristics and follow-up data of the enrolled GC patients were recorded. As shown in Table I, XTP8 level was positively correlated with lymphatic metastasis and distant metastasis of GC patients, rather than age, gender, and tumor staging. In addition, the Kaplan-Meier curves revealed poorer survival in GC patients with a high expression level of XTP8 (Figure 1C).

Knockdown of XTP8 Suppressed the Proliferation and Metastasis of GC

Three XTP8 shRNAs were constructed, and their transfection efficacies were all excellent in MKN-45 and SGC-7901 cells (Figure 1D, 1E). In the following experiments, sh-XTP8#3, the most pronounced one, was applied. Transfection with sh-XTP8#3 remarkably reduced viability in MKN-45 and SGC-7901 cells (Figure 2A, 2B). In addition, the silence of XTP8 attenuated the migration and invasion capacities of GC cells (Figure 2C, 2D).

Interaction Between XTP8 and TGIF1

Dual-Luciferase reporter gene assay was conducted to identify the interaction between XTP8 and TGIF1. The decline of the Luciferase activity after co-transfection of pcDNA-TGIF1 and pmirGLO-XTP8-WT supported the findings that TGIF1 was the target of XTP8 (Figure 3A). Transfection with sh-XTP8#3 upregulated TGIF1 level in GC cells, while the transfection with si-TGIF1 upregulated the XTP8 level (Figure 3B, 3C). A negative correlation was discovered between the expression levels of XTP8 and TGIF1 in GC tissues (Figure 3D).

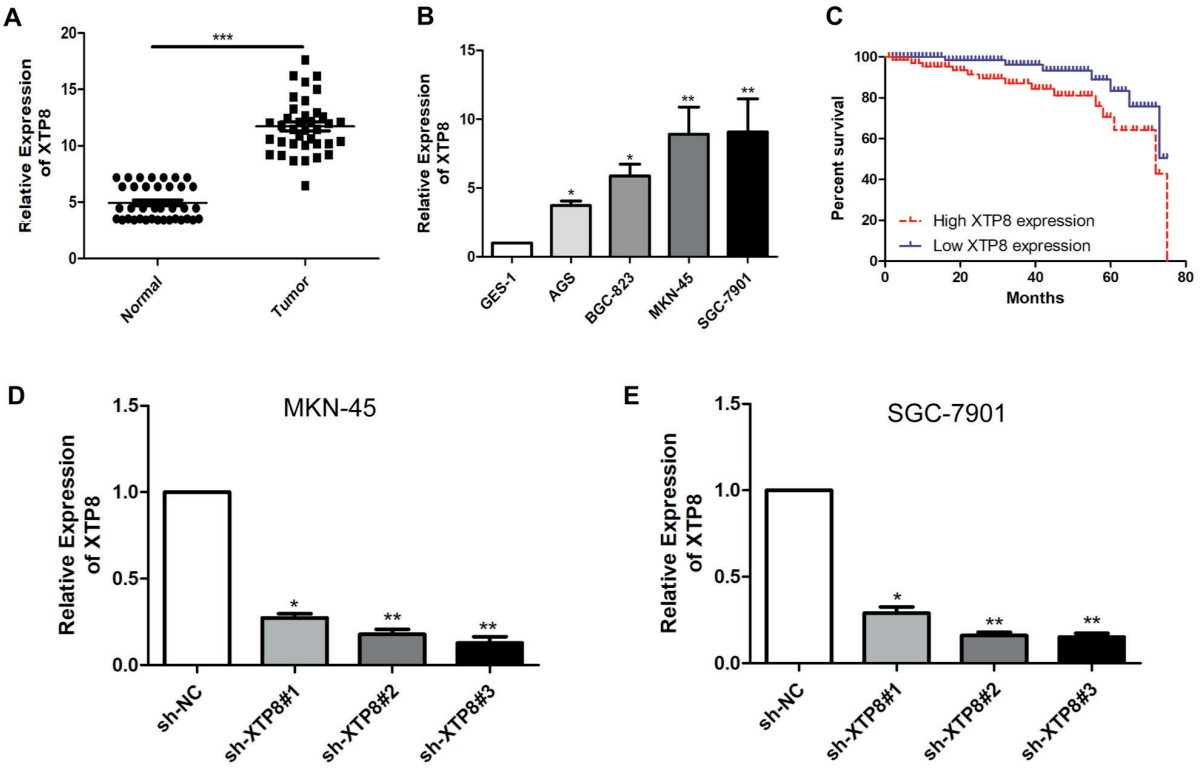

Figure 1. XTP8 is highly expressed in GC. **A,** XTP8 level in GC tissues and adjacent normal tissues. **B,** XTP8 level in human gastric mucosal cells and GC cells. **C,** Prognosis in GC patients with high and low expression levels of XTP8. **D-E,** Transfection efficacy of sh-XTP8#1, sh-XTP8#2 and sh-XTP8#3 in MKN-45 (**D**) and SGC-7901 cells (**E**).

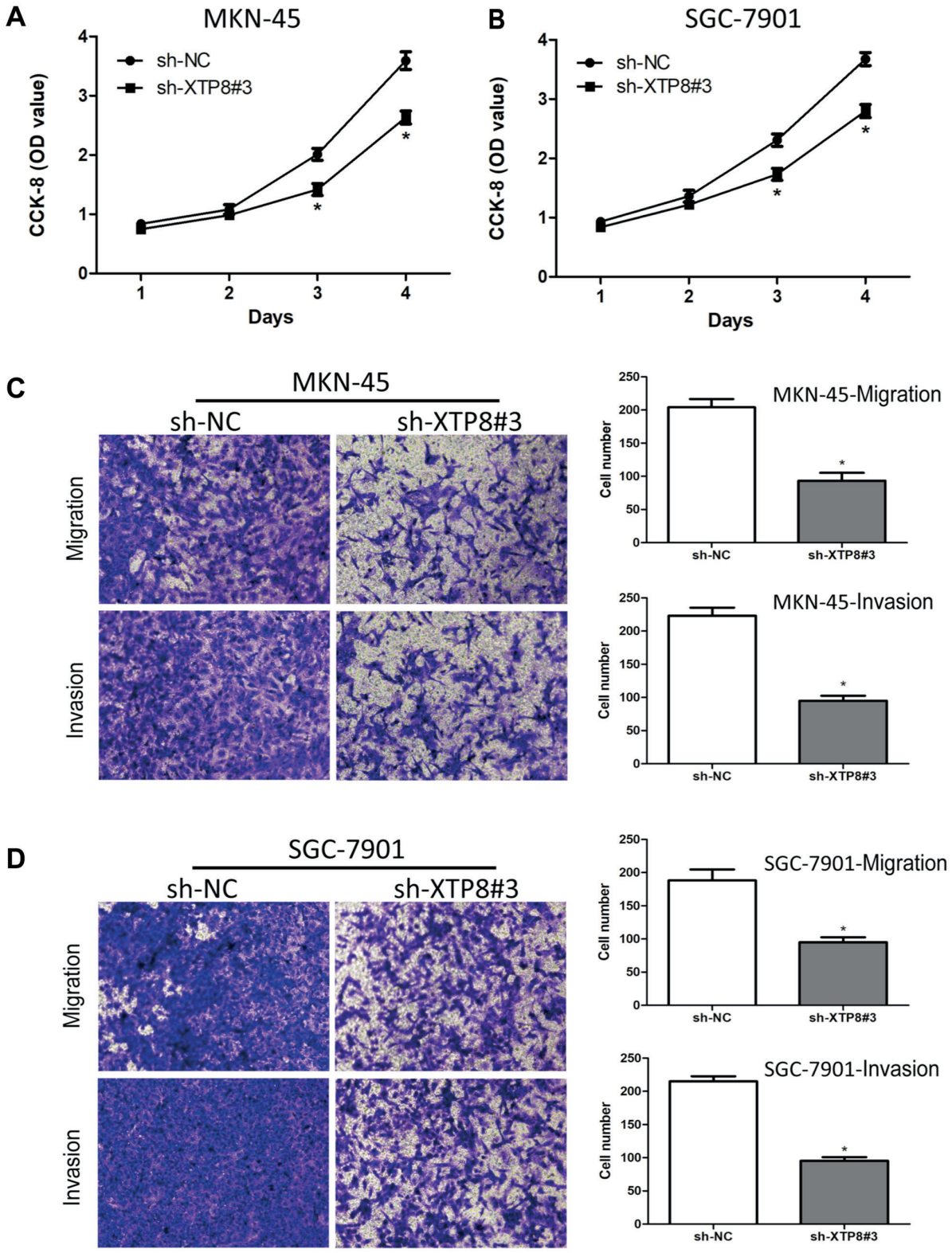

Figure 2. Knockdown of XTP8 suppresses the proliferation and metastasis of GC. **A-B,** Viability of MKN-45 (**A**) and SGC-7901 cells (**B**) transfected with sh-NC or sh-XTP8#3. **C-D,** Migration and invasion of MKN-45 (**C**) and SGC-7901 cells (**D**) transfected with sh-NC or sh-XTP8#3 (20×).

Table I. Association of XTP8 expression with clinicopathologic characteristics of gastric cancer.

Parameters	Number of cases	XTP8 expression		*p*-value
		Low (%)	High (%)	
Age (years)				0.419
<60	16	9	7	
≥60	21	9	12	
Gender				0.404
Male	17	9	8	
Female	20	9	11	
T stage				0.603
T1-T2	21	11	10	
T3-T4	16	7	9	
Lymph node metastasis				0.010
No	23	15	8	
Yes	14	3	11	
Distance metastasis				0.012
No	21	14	7	
Yes	16	4	12	

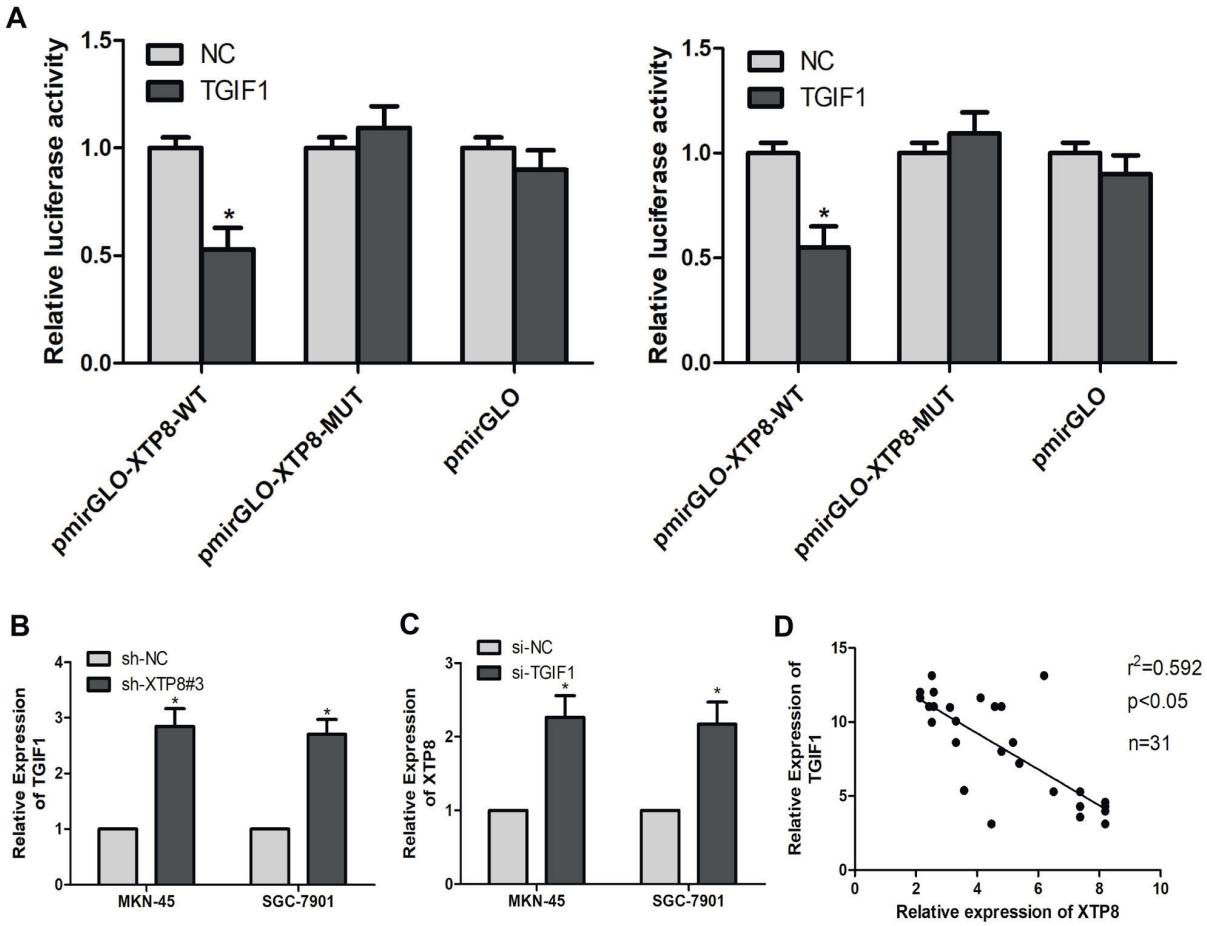

Figure 3. Interaction between XTP8 and TGIF1. **A,** Luciferase activity in MKN-45 and SGC-7901 cells co-transfected with pmirGLO-XTP8-WT/pmirGLO-XTP8-MUT/pmirGLO and NC/pcDNA-TGIF1. **B,** TGIF1 level in MKN-45 and SGC-7901 cells transfected with sh-NC or sh-XTP8#3. **C,** XTP8 level in MKN-45 and SGC-7901 cells transfected with si-NC or si-TGIF1. **D,** A negative correlation between XTP8 and TGIF1 in GC tissues.

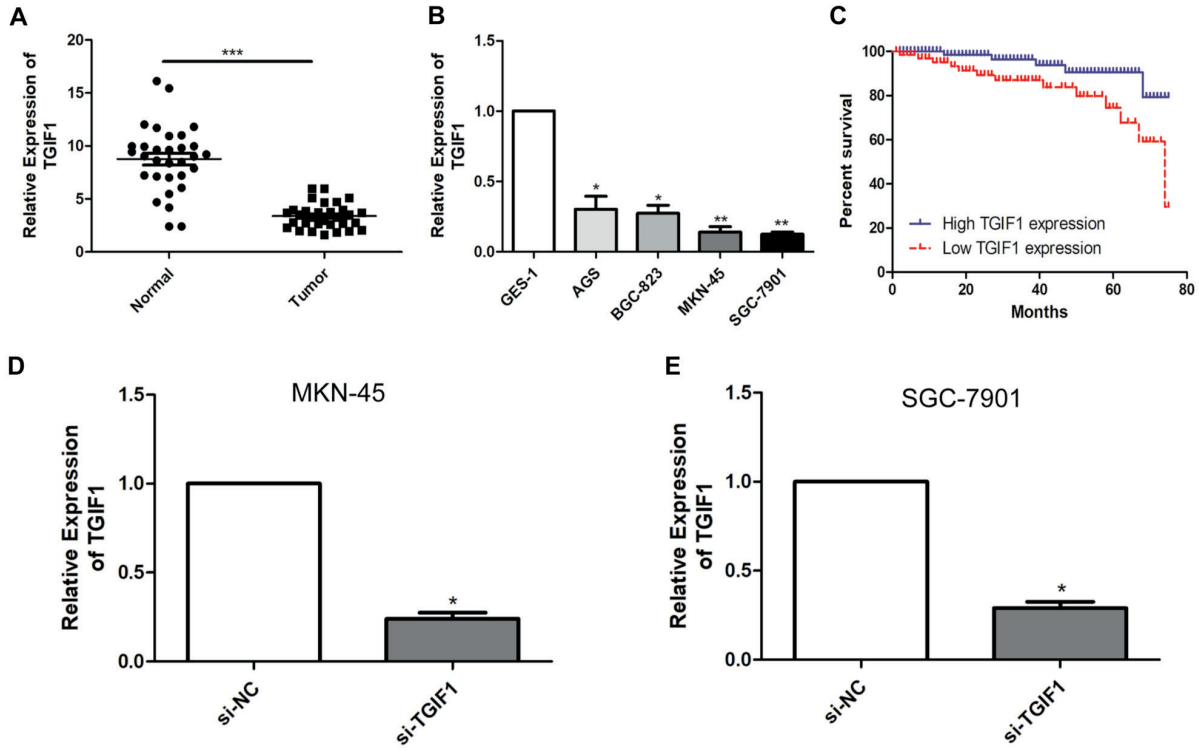

Figure 4. TGIF1 is lowly expressed in GC. **A,** TGIF1 level in GC tissues and adjacent normal tissues. **B,** TGIF1 level in human gastric mucosal cells and GC cells. **C,** Prognosis in GC patients with high and low expression levels of TGIF1. **D-E,** Transfection efficacy of si-TGIF1 in MKN-45 (**D**) and SGC-7901 cells (**E**).

TGIF1 Was Lowly Expressed in GC

TGIF1 was found to be lowly expressed in GC tissues and cells (Figure 4A, 4B). By analyzing the follow-up data, GC patients with a low expression level of TGIF1 suffered poorer survival (Figure 4C). Transfection with si-TGIF1 effectively downregulated TGIF1 level in MKN-45 and SGC-7901 cells, showing a satisfactory transfection efficacy (Figure 4D, 4E).

XTP8/TGIF1 axis regulated the progression of GC

It was speculated that TGIF1 was involved in XTP8-mediated progression of GC. Here, the downregulated level in GC cells transfected with sh-XTP8#3 was elevated after the co-transfection with si-TGIF1 (Figure 5A). Reduced viability of GC cells with XTP8 knockdown was abolished by the silence of TGIF1 (Figure 5B). Similarly, attenuated migration and invasion capacities in GC cells transfected with sh-XTP8#3 were partially reversed by the silence of TGIF1 (Figure 5C). Therefore, TGIF1 was responsible for the progression of GC affected by XTP8.

Discussion

Early-stage GC lacks evident signs and symptoms. The diagnosis of GC depends on gastroscopy and biopsy. Due to unbalanced economic development, gastroscopy screening is difficult to popularize in most regions, leading to an extremely low detection rate of early-stage GC[3-5]. Therefore, it is necessary to develop a cheap, effective, and sensitive approach for early screening of GC[5]. High rate of metastasis is a difficulty in the treatment of GC[13-15]. As a result, the elucidation of the mechanisms underlying metastatic GC is of clinical value[9,10].

Strong migration and invasion capacities are the most significant features of malignant tumors, which are also the leading causes of tumor death[14,15]. Tumor metastasis involves many processes,, such as cell adhesion, protease hydrolyzation of tissue matrix, and cell migration[19,20]. Cell motility is popular in normal physical progressions, such as tissue development, wound healing, angiogenesis, and it is also involved in tumorigenesis and other pathological activities[18].

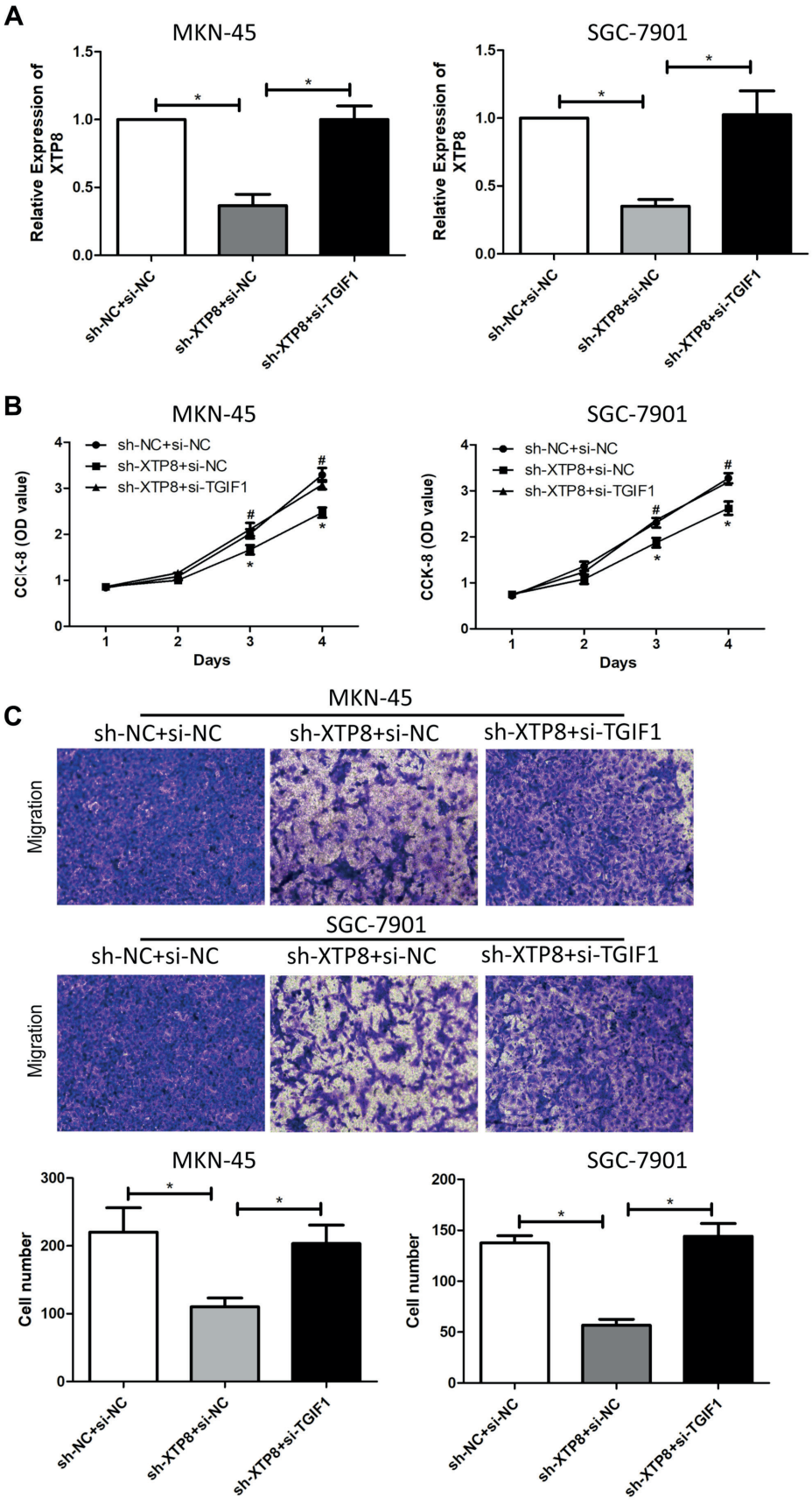

Figure 5. XTP8/TGIF1 axis regulates the progression of GC. MKN-45 and SGC-7901 cells are transfected with sh-NC, sh-XTP8#3+si-NC, or sh-XTP8#3+si-TGIF1, respectively. **A,** XTP8 level. **B,** Viability. **C,** Migration and invasion (20×).

During the migration process, dynamic assembly of intracellular cytoskeletal proteins, the changes in cell adhesion and extracellular matrices and remodeling of surrounding matrices are all regulated by complicated pathways[21,22]. In this study, XTP8 was found to be markedly upregulated in GC tissues and cells. In addition, XTP8 level was positively related to metastasis and prognosis of GC patients. Therefore, it was believed that XTP8 acted as an oncogene in GC. Furthermore, the silence of XTP8 was able to attenuate proliferation and metastasis capacities of MKN-45 and SGC-7901 cells.

Through bioinformatics and Dual-Luciferase reporter gene assay, a potential interaction between XTP8 and TGIF1 was identified. XTP8 negatively regulated TGIF1 level in GC tissues, and the silence of TGIF1 markedly accelerated GC cells to proliferate, migrate, and invade. Notably, TGIF1 was discovered to be necessary during the progression of GC affected by XTP8. The transcriptional activity of the XTP8 locus may be regulated by TGIF1.

Conclusions

XTP8 is upregulated in GC, and closely linked to lymphatic metastasis, distant metastasis, and poor prognosis of GC patients. Besides, it accelerates the malignant progression *via* negatively regulating TGIF1.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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