

Knockdown of lncRNA TP73-AS1 inhibits gastric cancer cell proliferation and invasion via the WNT/ β -catenin signaling pathway

YUFENG WANG, SHUAI XIAO, BINGYI WANG, YANG LI and QUANNING CHEN

Department of General Surgery, Tongji Hospital, Tongji University School of Medicine, Shanghai 200065, P.R. China

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Abstract. Long non-coding RNAs (lncRNAs) function as tumor suppressors or oncogenes in tumor development and progression. The purpose of the present study was to investigate the clinical significance and functional role of lncRNA TP73-AS1 in gastric cancer (GC). Reverse transcription-quantitative polymerase chain reaction analysis demonstrated that TP73-AS1 was significantly upregulated in GC tissues compared with adjacent normal tissues. The higher expression of TP73-AS1 was closely associated with lymph node metastasis and tumor-node-metastasis stage in patients with GC. Those patients with GC showing a higher expression of TP73-AS1 were predicted to have shorter disease-free survival and overall survival rates. The knockdown of TP73-AS1 was shown to markedly inhibit cell proliferation, cell colony formation and cell invasion. In addition, the downregulation of TP73-AS1 suppressed the expression of transcription factor 4 and β -catenin, which suggested that a decrease in TP73-AS1 suppressed the WNT/ β -catenin signaling pathway in GC. Therefore, these results indicated that TP73-AS1 may be a target for GC treatment.

Introduction

Gastric cancer (GC) is the second most common cause of cancer-associated mortality worldwide. There are ~100,000 new cases of GC annually, and >700,000 cases of GC-associated mortality (1). It is reported that ~50% of patients with GC exhibit metastasis at the time of diagnosis (2). Therefore, it is important to identify novel early diagnostic markers and therapeutic targets of tumor invasion and metastasis.

Long non-coding RNAs (lncRNAs) have been identified as a class of non-protein-coding transcripts, which are 200 nucleotides in length. Previous studies have reported that the abnormal expression of lncRNAs is significantly involved in cancer development and progression (3,4). In GC, the overexpression of lncRNA SPRY4-IT1 promotes tumor cell proliferation and invasion by activating enhancer of zeste homolog 2 in hepatocellular carcinoma (HCC) (5). The decreased expression of lncRNA LINC00261 predicts a poor prognosis in patients with GC and suppresses tumor metastasis by affecting the epithelial-mesenchymal transition (6). The overexpression of metastasis-associated lung adenocarcinoma transcript 1 promotes cell invasion and metastasis of GC by increasing the expression of epidermal growth factor-like domain 7 (7). The increased expression of lncRNA taurine upregulated 1 also predicts a poor prognosis of GC and regulates cell proliferation by epigenetically silencing p57 (8).

Previous studies have shown that TP73-AS1 is involved in tumor progression. For example, the silencing of lncRNA TP73-AS1 inhibits cell proliferation and induces apoptosis in esophageal squamous cell carcinoma (9). The TP73-AS1 interacts with microRNA (miR)-142 to modulate brain glioma growth through the high mobility group box 1 (HMGB1)/receptor for advanced glycation end products (RAGE) pathway (10). lncRNA TP73-AS1 modulates HCC cell proliferation through miR-200a-dependent HMGB1/RAGE regulation (11); however, the expression and molecular mechanism underlying lncRNA TP73-AS1 in GC remains to be elucidated.

In the present study, it was shown that TP73-AS1 was upregulated in GC tissues. The higher expression of TP73-AS1 was associated with poor prognosis in patients with GC. The downregulation of TP73-AS1 suppressed cell proliferation and invasion. Additionally, the knockdown of TP73-AS1 suppressed the WNT/ β -catenin signaling pathway in GC. These results indicated that TP73-AS1 may be a target for GC treatment.

Materials and methods

Patient tissue specimens. A total of 64 paired human GC tissue specimens and corresponding adjacent normal tissues were obtained from patients who underwent surgical resection between January 2010 and March 2013 in the Department

Correspondence to: Dr Quanning Chen, Department of General Surgery, Tongji Hospital, Tongji University School of Medicine, 389 Xincun Road, Putuo, Shanghai 200065, P.R. China
E-mail: chenquanningw@126.com

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of General Surgery of Shanghai Tongji Hospital (Shanghai, China). All GC tissue specimens were immediately snap-frozen in liquid nitrogen until total RNA extraction. Informed consent was obtained from all patients and the study was approved by the Medical Ethics Committee of Shanghai Tongji Hospital.

Cell culture. The human GC, MGC-803, the BGC-823 cell lines, and a normal gastric epithelium cell line (GES-1) were purchased from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). All cells were cultured in DMEM supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 5% CO₂ at 37°C.

Cell transfection. The small interfering RNA si-TP73-AS1-1 sense, 5'-GATCGCGTTCTGTGTGGAAGTTACTGGATCAAGAGTCCAGTAAGTTCCACACAGAATTTTTTCCAA A-3' and antisense, 5'-AGCTTTTGGAAAAAATTCTGTGTGGAAGTTACTGGACTCTTGATCCAGTAAGTTCCA CACAGAACGC-3'; si-TP73-AS1-2 sense, 5'-GATCGCGTA GACAGAGGTCATCAGCCAGTCAAGAGCTGGCTGATG ACCTCTGTCTATTTTTTCCAAA-3' and antisense, 5'-AGC TTTTGGAAAAAATAGACAGAGGTCATCAGCCAGCTC TTGACTGGCTGATGACCTCTGTCTACGC-3'; and si-negative control (NC) were synthesized and purchased from GenePharma Co. Ltd. (Shanghai, China). Cell transfection was performed using Lipofectamine™ 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The GC tissues and cells were used to extract total RNA using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. The RNA was measured using the Nano Drop 2000 Spectrophotometer (Nano Drop Technologies; Thermo Fisher Scientific, Inc., Wilmington, DE, USA) and the ratio of absorbance was at 260 to 280 nm (260/280 ratio). The 1 µg RNA samples were transcribed to cDNA using a First Strand cDNA Synthesis kit (Tiangen Biotech Co., Ltd., Beijing, China). The reverse transcription reaction condition was as follows: 37°C for 15 min, 85°C for 5 sec and 4°C for 2 min. The mRNA was detected using TaqMan MicroRNA PCR kit (Takara Biotechnology Co., Ltd., Dalian, China) on ABI 7500 fast system (Applied Biosystems; Thermo Fisher Scientific, Inc., Carlsbad, CA, USA). To quantify the mRNA expression, the SYBR Premix Ex Taq (Takara Biotechnology Co., Ltd., Dalian, China) was used to amplifying the mRNA expression. The qRT-PCR reaction conditions were as follows: 95°C for 30 sec, followed by 40 cycles at 95°C for 5 sec and 60°C for 34 sec. GAPDH was used as the internal control. The RT-qPCR results were calculated using the 2^{-ΔΔC_q} method (12). The primer sequences were as follows: LnRNATP73-AS1 forward, 5'-CCG GTTTTCCAGTTCTTGAC-3' and reverse, 5'-GCCTCACAG GGAAACTTCATGC-3'; GAPDH forward, 5'-GGTGAAGGT CGGAGTCAACG-3' and reverse, 5'-CAAAGTTGTCATGGA TGHACC-3'.

Cell proliferation and cell colony formation assay. Cell proliferation was evaluated with Cell Counting kit-8 (CCK-8) solution (Dojindo Molecular Technologies, Inc., Kumamoto,

Japan) in accordance with the manufacturer's protocol. Briefly, the MGC-803 and BGC-823 cells (2x10³ cells/well) were seeded into 96-well plates in triplicate. The proliferative activity of cells was detected at 0, 24, 48, and 72 h, and 10 µl of CCK-8 solution was added to each well and cultured for 2 h at room temperature. Subsequently, cellular proliferation was detected at the absorbance of the converted dye at 450 nm. For the cell colony assay, the MGC-803 and BGC-823 cells (200 cells/well) were seeded into 6-well plates in triplicate. The cells were cultured for 3 weeks at 37°C in 5% CO₂. The cells were then fixed with 100% methanol for 20 min at room temperature and stained with 0.1% crystal violet for 10 min at room temperature, and the numbers of cell colonies were calculated under a light microscope (DP72; Olympus Corporation, Tokyo, Japan).

Cell invasion assay. The cell invasion assays were performed using Transwell chambers with a pore size of 8-µm (Corning Incorporated, Cambridge, MA, USA) and coated with Matrigel (BD Biosciences, San Jose, CA, USA). A total of 5x10⁴ transfected MGC-803 and BGC-823 cells were resuspended in 300 µl of medium and seeded in the upper chamber, with 500 µl of medium supplemented with 10% FBS added to the lower chamber. Following culture of the cells for 24 h, the cells in the lower chamber were fixed with methanol for 10 min (Beyotime Institute of Biotechnology, Shanghai, China) and stained with 0.1% crystal violet (Beyotime Institute of Biotechnology) for 10 min. The cells were counted under an inverted microscope in five randomly selected fields (Olympus Corporation, Tokyo, Japan).

Western blot analysis. The cells were lysed with RIPA buffer (Beyotime Institute of Biotechnology). Protein quantity was detected using a bicinchoninic acid assay kit (Beyotime Institute of Biotechnology), according to the manufacturer's protocol. The cell protein lysates (40 µg) were separated via 10% SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes (EMD Millipore, Billerica, MA, USA). The PVDF membranes were blocked with 5% skimmed milk for 2 h at room temperature. The PVDF membranes were then incubated with the following specific antibodies: Transcription factor 4 (TCF4; cat. no. 2566; 1:1,000 dilution); β-catenin (cat. no. 9562; 1:1,000 dilution); and GAPDH (cat. no. 5174; 1:3,000 dilution; all Cell Signaling Technology, Inc., Beverly, MA, USA) overnight at 4°C. Subsequently, the secondary antibody (goat anti-rabbit IgG; cat. no. sc-2004; 1:1,000 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was added to the PVDF membranes and for incubated for 1.5 h at room temperature. The blots were determined using a chemiluminescence detection kit (GE Healthcare Life Sciences, Piscataway, NJ, USA). The gray bands were analyzed using Image J software (version 1.46; National Institutes of Health, Bethesda, MD, USA). The GAPDH protein expression was used as the internal control.

Statistical analysis. Statistical analysis was performed using SPSS software (version 17.0; SPSS, Inc., Chicago, IL, USA). All data are shown as the mean ± standard deviation. Comparison of means between different samples was analyzed using Student's t-test and one-way analysis of variance. P<0.05 was considered to indicate a statistically significant difference.

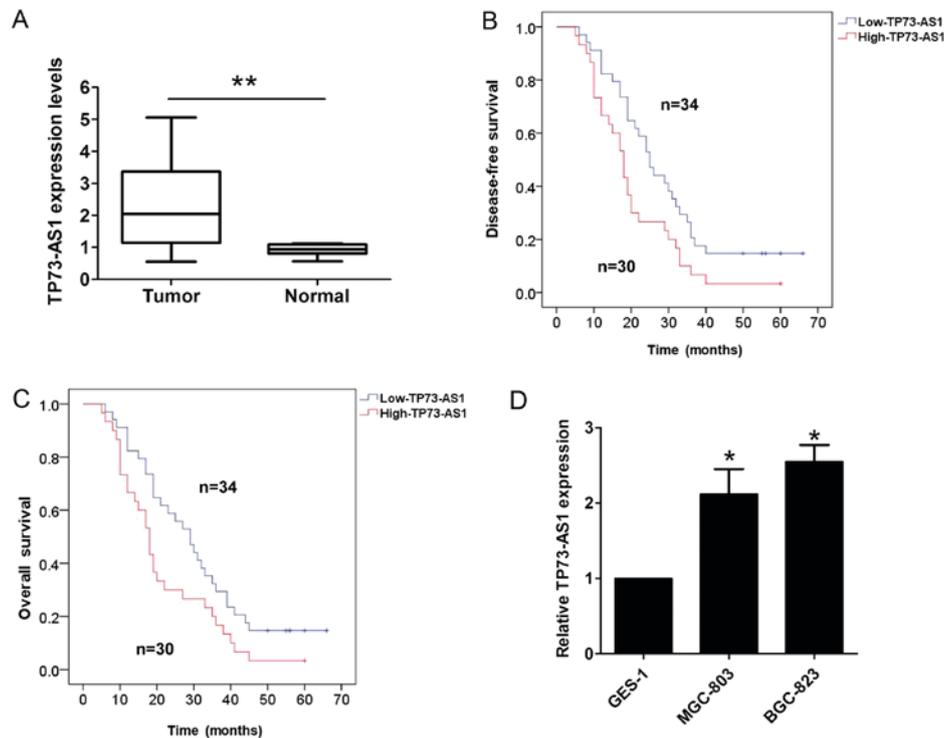


Figure 1. Expression of TP73-AS1 is significantly upregulated in GC tissues compared with adjacent normal tissues. (A) TP73-AS1 was significantly upregulated in 64 pairs of GC tissues when compared with adjacent normal tissues using reverse transcription-quantitative polymerase chain reaction analysis. (B) Disease-free survival rate was statistically higher in the lower TP73-AS1 expression group, compared with that in the higher TP73-AS1 expression group in the 64 GC patients. (C) Overall survival rate was statistically higher in the lower TP73-AS1 expression group, compared with that in the higher TP73-AS1 expression group in the 64 GC patients. (D) TP73-AS1 expression was higher in MGC803 and BGC-823 cells than that in GES-1 cells. * $P < 0.05$, ** $P < 0.01$. GC, gastric cancer.

Results

Expression of TP73-AS1 is upregulated in GC tissues. The present study first detected the expression of TP73-AS1 in 64 paired human GC tissue specimens and corresponding adjacent normal tissues using RT-qPCR analysis. As shown in Fig. 1A, the expression of TP73-AS1 was higher in the GC tissues than the corresponding adjacent normal tissues. Furthermore, the association between the expression of TP73-AS1 and clinicopathologic factors in patients with GC was evaluated. The results of the statistical analysis showed that a higher expression of TP73-AS1 was correlated with lymph node metastasis ($P = 0.001$) and tumor-node-metastasis (TNM) stage ($P = 0.003$; Table I); however, no association existed with other clinicopathologic features, including sex, age and tumor size ($P > 0.05$; Table I). The results of the Kaplan-Meier analysis indicated that a higher expression of TP73-AS1 in patients with GC predicted a shorter disease-free survival (DFS; log rank=5.412, $P < 0.05$; Fig. 1B) and overall survival (OS; log rank=4.506, $P < 0.05$; Fig. 1C). In addition, it was demonstrated that the expression of TP73-AS1 was significantly higher in two GC cell lines (MGC-803 and BGC-823), compared with that in the normal gastric epithelium cell line (GES-1; Fig. 1D).

Downregulation of the expression of TP73-AS1 decreases cell proliferation in GC. To clarify the functional effects of the expression of TP73-AS1 on cell proliferation, CCK-8 and cell colony formation assays were performed. First, two siRNAs against TP73-AS1 were applied to knock down TP73-AS1 in MGC-803 and BGC-823 cells. The results

showed that si-TP73-AS1-2 had a higher silencing efficiency than TP73-AS1-1, and was selected for use in the following experiments (Fig. 2A and B). The results of the CCK-8 assays showed that the cell proliferation rate was significantly reduced at 48 and 72 h in the MGC-803 and BGC-823 cells following knockdown (Fig. 2C and D). Additionally, the number of cell colonies following cell transfection with si-TP73-AS1 on day 14 was markedly reduced in the MGC-803 and BGC-823 cells (Fig. 3A and B). These results showed that the downregulation of TP73-AS1 decreased the proliferation of GC cells.

Downregulation of the expression of TP73-AS1 decreases the invasion of GC cells. To detect the effects of the expression of TP73-AS1 on cell invasion, Transwell invasion assays were performed. Following cell transfection with si-NC or si-TP73-AS1 for 48 h, the cell invasion was significantly decreased, compared with that in the si-NC group in the MGC-803 and BGC-823 cells (Fig. 3C and D). Therefore, downregulation of the expression of TP73-AS1 decreased cell invasion in GC.

Downregulation of the expression of TP73-AS1 inhibits the WNT/ β -catenin signaling pathway in GC cells. According to the above findings, a higher level of TP73-AS1 contributed to the progression of GC. The present study examined the potential mechanism by which TP73-AS1 regulates GC cell proliferation and invasion. The WNT/ β -catenin signaling pathway is significantly associated with GC cell proliferation and invasion (13). Following cell transfection with si-NC or si-TP73-AS1 for 48 h, the downregulated expression of

Table I. Association between the expression of TP73-AS1 and the clinicopathological factors of 64 patients with gastric cancer.

Clinicopathologic factor	Patients (n)	Expression of TP73-AS1		P-value
		Lower (n=34)	Higher (n=30)	
Sex				0.223
Male	42	20	22	
Female	22	14	8	
Age (years)				0.934
≤55	43	23	20	
>55	21	11	10	
Tumor size				0.924
<3 cm	38	20	18	
>3 cm	26	14	12	
Histological differentiation				0.375
High, middle	39	23	16	
Low	25	11	14	
Lymph node metastasis				0.001 ^a
No	36	26	10	
Yes	28	8	20	
Local invasion				0.090
T1-T2	37	23	14	
T3-T4	27	11	16	
TNM stage				0.003 ^a
I/II	38	26	12	
III/IV	26	8	18	

^aP<0.05 was considered statistically significant. TNM, tumor-node-metastasis.

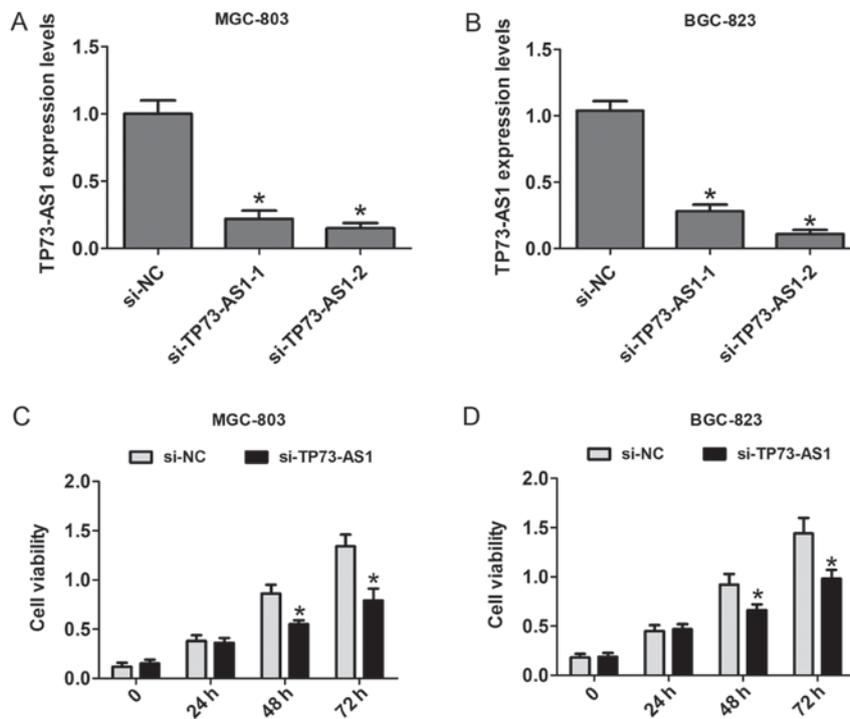


Figure 2. Downregulation of the expression of TP73-AS1 inhibits cell proliferation ability in gastric cancer. Expression of TP73-AS1 was significantly downregulated following transfection of cells with si-TP73-AS1-1 or si-TP73-AS1-2 in (A) MGC-803 and (B) BGC-823 cells. Cell viability was detected at 0, 24, 48 and 72 h when cells were transfected with si-NC or si-TP73-AS1 in (C) MGC-803 and (D) BGC-823 cells. *P<0.05, vs. si-NC. si, small interfering RNA; NC, negative control.

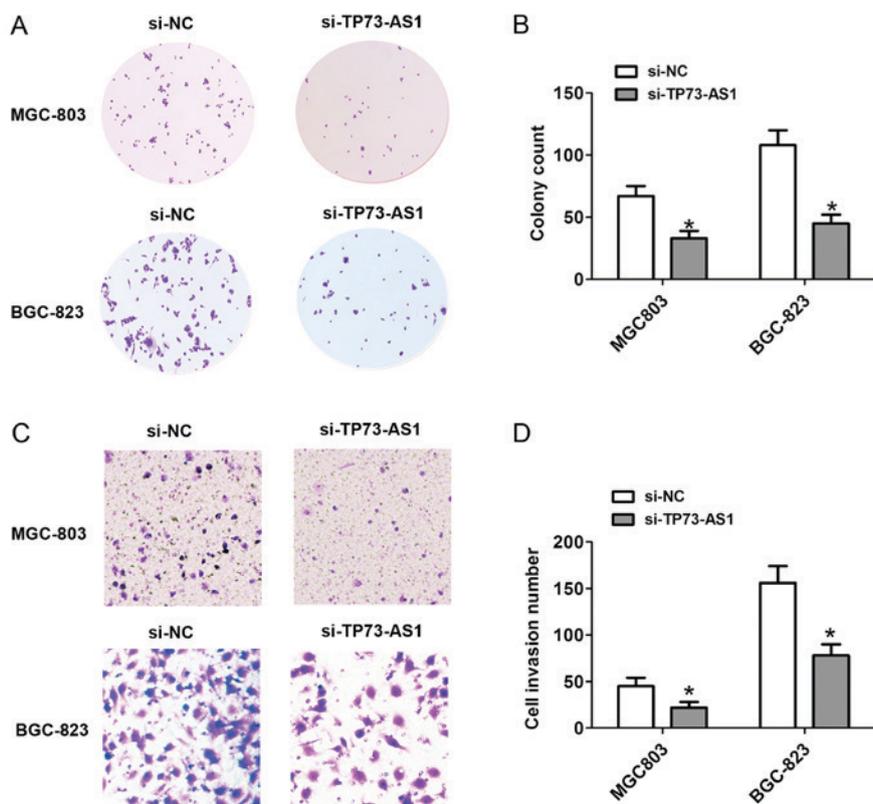


Figure 3. Downregulation of the expression of TP73-AS1 inhibits cell colony formation and cell invasion in gastric cancer. (A) Images of colonies; (B) numbers of cell colonies were calculated at 48 h when the cells were transfected with si-NC or si-TP73-AS1 in MGC-803 or BGC-823 cells. Magnification, x200. (C) Images of the cell invasion assay; (D) numbers of invading cells were evaluated at 48 h when cells were transfected with si-NC or si-TP73-AS1 in MGC-803 or BGC-823 cells, Magnification, x200. * $P < 0.05$, vs. si-NC. si, small interfering RNA; NC, negative control.

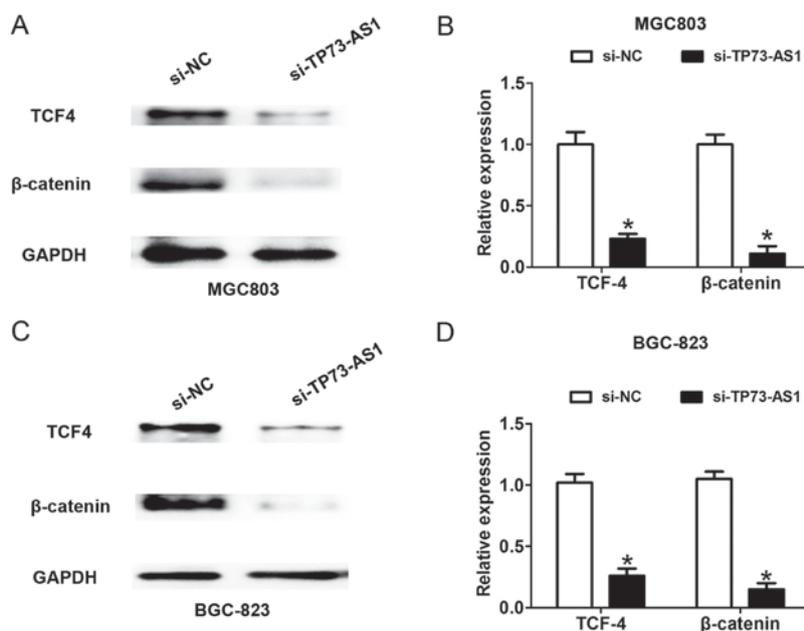


Figure 4. Downregulated expression of TP73-AS1 inhibits the WNT/ β -catenin signaling pathway in gastric cancer cells (A) Images of blots were captured and (B) relative protein expression was evaluated at 48 h when cells were transfected with si-NC or si-TP73-AS1 in MGC-803 cells. (C) Images of blots were captured and (D) relative protein expression was evaluated at 48 h when cells were transfected with si-NC or si-TP73-AS1 in BGC-823 cells. * $P < 0.05$, vs. si-NC. TCF4, transcription factor 4; si, small interfering RNA; NC, negative control.

TP73-AS1 suppressed the protein expression of TCF4 and β -catenin in the MGC-803 and BGC-823 cells (Fig. 4A-D),

which suggested that the knockdown of TP73-AS1 suppressed the WNT/ β -catenin signaling pathway in GC.

Discussion

Deregulation of the expression of lncRNA in various types of cancer is essential for tumorigenesis and development. Previously, studies have demonstrated that lncRNAs may be applied to serve as prognostic markers and therapeutic targets in tumors. In GC, the lncRNA, highly upregulated in liver cancer, acts as a novel serum biomarker for the diagnosis and prognostic prediction of GC (14). The overexpression of lncRNA homeobox A transcript at the distal tip enhances tumor invasion and predicts poor prognosis in GC (15). The plasma level of H19 is also significantly higher in patients with GC and serves as a potential biomarker for diagnosis (16). The decreased expression of the lncRNA LINC00261 indicates poor prognosis in GC and suppresses GC metastasis by affecting the epithelial-mesenchymal transition (6). In the present study, it was shown that TP73-AS1 was upregulated in GC tissues compared with adjacent normal tissues. The higher expression of TP73-AS1 was associated with lymph node metastasis, TNM stage, and poor DFS and OS rates in patients with GC. Therefore, TP73-AS1 functions as a biomarker for predicting the prognosis of patients with GC.

Previous studies have indicated that decreased TP73-AS1 inhibits cell proliferation and induces apoptosis in esophageal squamous cell carcinoma (9). The knockdown of TP73-AS1, an lncRNA of one triplet, not only reduced the expression of regulatory factor X1, an mRNA of this triplet, but also induced apoptosis in U87 cells (17). In the present study, it was demonstrated that the knockdown of TP73-AS1 suppressed cell proliferation, cell colony formation and cell invasion, which suggested that TP73-AS1 exerts a tumor-promoting function in GC cells. Studies have found that the Wnt/ β -catenin signaling pathway is involved in oncogenesis, including the initiation and progression of GC (18). Wnt/ β -catenin signaling enhances the transcriptional activity of cyclooxygenase-2 in GC cells (19), and aquaporin 3 promotes the stem-like properties of GC cells via the Wnt/glycogen synthase kinase-3 β / β -catenin pathway (20). In the present study, downregulation of the expression of TP73-AS1 suppressed the expression of TCF4 and β -catenin in GC cells, which suggested that the reduction in TP73-AS1 suppressed the WNT/ β -catenin signaling pathway in GC. These results indicated that TP73-AS1 promotes cell proliferation and invasion via regulation of the WNT/ β -catenin signaling pathway in GC.

In conclusion, the present study showed that the expression of TP73-AS1 was higher in GC, and that the knockdown of TP73-AS1 suppressed cell proliferation and invasion. Furthermore, the knockdown of TP73-AS1 suppressed the WNT/ β -catenin signaling pathway in GC. These results indicated that TP73-AS1 may be a therapeutic target for GC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YW, BW and SX analyzed and interpreted the patient data. QC and YL conceived and designed the study. YW, BW and SX performed the experiments. QC drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Medical Ethics Committee of Shanghai Tongji Hospital and informed consent was obtained from all participants.

Patient consent for publication

Informed consent was obtained from all patients.

Competing interests

The authors declare that they have no competing interests.

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