E2F6 promotes cell proliferation and invasion in gastric cancer through the regulation of IncRNA TUSC7

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Original Research Article

Abstract

Purpose: To examine the regulatory effects of E2F6/long noncoding ribonucleic acid (lncRNA) TUSC7 on the progression of gastric cancer (GC), and the underlying mechanism of action.

Methods: Relative levels of E2F6 and TUSC7 in GC tissues were determined by quantitative real-time polymerase chain reaction (qRT-PCR). The influence of TUSC7 on the overall survival of patients with GC was analyzed using Kaplan-Meier method. Interaction between E2F6 and TUSC7 was analyzed by Spearman correlation test and dual-luciferase reporter gene assay. Changes in proliferation, invasion and apoptosis of BGC823 and SGC7901 cells regulated by E2F6 and TUSC7 were assessed. The protein levels of MMP-2 and the activity of caspase-3 were evaluated in GC cells treated with E2F6 and TUSC7 using western blot and caspase-3 kit.

Results: TUSC7 was downregulated while E2F6 was upregulated in GC tissues and cells. A negative correlation was observed between the expression levels of TUSC7 and E2F6, and a low level of TUSC7 predicted poor prognosis in GC patients. The knockdown of E2F6 attenuated cell viability and invasion, and stimulated the apoptosis of GC cells, but these changes were partially reversed by co-knockdown of TUSC7. In addition, knockdown of E2F6 downregulated MMP-2 protein levels, and upregulated caspase-3 in GC cells, but these changes were partially reversed by co-transfection of si-TUSC7.

Conclusion: TUSC7 is downregulated in GC, and its low level predicts poor prognosis in GC patients. E2F6 promotes proliferation and invasion of GC cells by negatively regulating TUSC7. Therefore, E2F6/TUSC7 may be utilized as potential therapeutic targets for GC.

Keywords: Gastric cancer, Cell proliferation and invasion, IncRNA TUSC7, E2F6

INTRODUCTION

Gastric cancer (GC) is a highly prevalent tumor in the digestive tract. Its incidence ranks fourth of all malignancies. Owing to high rates of recurrence and metastasis, GC has become the second leading cause of tumor death [1]. The incidence of GC worldwide varies a lot, based on gender and region. It is reported that the incidence of GC in males is about twice that of females. Over 70% of GC cases occur in developed countries. In particular, the incidence of GC is extremely high in East Asia, Eastern Europe and Southern United States. Currently, dietary habits, H. pylori infection and heredity are considered to be the major pathogenic factors of
GC [2]. The occurrence and progression of GC is very complicated. Endoscopic or surgical resection of early-stage GC leads to a good prognosis. Nevertheless, the detection rate of early-stage GC is very low because of atypical symptoms and signs. Middle-stage or advanced GC is usually combined with lymphatic or distant metastasis, leading to a poor prognosis with the 5-year survival of 30 %. Tumor cell invasion and metastasis are the key events leading to tumor death [3,4]. Therapeutic targets for predicting and inhibiting tumor metastasis urgently need to be developed. With in-depth studies, epigenetics in GC has attracted great interest. Genome sequencing has proposed that protein-encoding genes only account for 2% of all genomes, and the majority is non-coding RNAs [5]. Accumulated evidence has demonstrated the regulatory effects of long non-coding RNAs (lncRNAs) on transcription, post-transcription and epigenetics, thereby influencing tumor diseases [6,7]. Tumor suppressor candidate gene 7 (TUSC7) is a novel lncRNA regulated by p53. Its diverse functions have emerged in studies of tumor diseases. For instance, TUSC7 suppresses proliferative and metastatic abilities in endometrial cancer by absorbing miR-616 to regulate SOCS4 (SOCS5) [8]. TUSC7 has been found to mitigate the progression of pancreatic cancer by regulating miR-371a-5p [9]. LncRNA-TUSC7/miR-224 regulatory loop affects chemotherapy-resistance in esophageal squamous cell carcinoma by competitively regulating DESC1 [10]. Knockdown of TUSC7 stimulates non-small cell lung cancer (NSCLC) to proliferate and its low level predicts poor prognosis [11]. We investigated the potential roles of E2F6/TUSC7 in the progression of GC.

METHODS

Tissue collection

A total of 60 paired GC tissues and adjacent normal tissues were surgically resected from GC patients admitted in Changzhou First People’s Hospital. All samples were pathologically confirmed and preserved in liquid nitrogen. The patients and their families in this study all received complete information. This study was approved by the Ethics Committee of Changzhou First People’s Hospital (approval no. 2022-CL068) and complied with international guidelines for human studies [12].

Cell culture

Gastric epithelial cells (GES1) and GC cells (AGS, BGC823, SGC7901 and MGC803) were provided by American type culture collection (ATCC, Manassas, VA, USA). Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640, Hyclone, South Logan, UT, USA) containing 10 % fetal bovine serum (FBS, Hyclone, South Logan, UT, USA), and 1 % penicillin-streptomycin in a 5 % CO2 incubator at 37 °C. Medium was regularly replaced. Cell passage was conducted at 80-90 % confluence.

Cell transfection

An overexpression vector of E2F6 was constructed using the pSicoR lentivirus vector and was amplified in 293T cells to produce LV-E2F6. Additionally, a negative control lentivirus (LV-NC) was also constructed. Following transfection of LV-E2F6 or LV-NC, the cells were incubated with puromycin for a duration of 2 weeks and subsequently, positive cells were selected. Transfection of si-E2F6 or si-TUSC7 (GenePharma, Shanghai, China) was conducted by Lipofectamine™ 3000 (Invitrogen, Carlsbad, CA, USA).

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Cells were lysed using the TRIzol method from Invitrogen (Carlsbad, CA, USA) to harvest RNAs, and the extracted RNAs were reversely transcribed to CDNA following the instructions of PrimeScript RT reagent Kit (TaKaRa, Tokyo, Japan). RNA concentration and purity were determined spectrophotometrically, and qualified RNA was applied for qRT-PCR using the SYBR Premix Ex Taq TM kit (TaKaRa, Tokyo, Japan). Glyceraldheyde 3-phosphate dehydrogenase (GAPDH) and U6 were used as internal references. Relative level was calculated using 2^{-∆∆CT} method. The primer sequences are detailed in Table 1.

Cell counting kit-8 (CCK-8) assay

Cells were seeded into 96-well plates with 1 × 10^3/mL. At the appointed time points, 10 µL of CCK-8 solution (Dojin, Kumamoto, Japan) was added to each well. The absorbance at 450 nm of each sample was measured by a microplate reader (Bio-Rad, Hercules, CA, USA).

Transwell assay

The cells were inoculated in a 24-well plate, and 200 µL of suspension (1 × 10^5/mL) was applied to the upper area of a Transwell chamber (Millipore, Billerica, MA, USA) and pre-coated with Matrigel.

Gastric epithelial cells (GES1) and GC cells (AGS, BGC823, SGC7901 and MGC803) were
In the bottom area, 500 μL of medium containing 10 % FBS was added. After 48 h of incubation, the penetrated cells in the bottom area were fixed in methanol for 15 min, dyed with crystal violet for 20 min and counted using a microscope. Invasive cell number was counted in 5 randomly selected fields per sample (magnification, 20×).

Flow cytometry

The cells were washed twice with phosphate-buffered saline (PBS) and then digested to prepare a cell suspension with a concentration of 1 × 10⁵ cells/mL. Thereafter, the cells were placed in 1 mL of pre-chilled 70 % ethanol and kept at 4 °C overnight. The following day, the cells were washed twice with PBS and incubated with 100 μL of RNaseA at 37 °C in the dark for 30 min, using a 37 °C water bath. Subsequently, the cells were dyed with 500 μL of PI at 4 °C in the dark for 15 min. Finally, the apoptotic rate was determined spectrophotometrically at a wavelength of 488 nm.

Dual-luciferase reporter gene assay

Wild-type or mutant-type TUSC7 was cloned into pGL3 vector. Cells were inoculated in a 24-well plate with 1 × 10⁵/mL suspension. After adherence, cells were co-transfected with wild-type/mutant-type TUSC7 and E2F6 vector/negative control for 24 h, followed by determination of luciferase activity (Promega, Madison, WI, USA).

Western blot

To determine protein concentration, cellular protein was extracted and loaded onto a dodecyl sulfate-sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE). The resulting gel was transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore in Billerica, MA, USA). The membranes were then blocked using 5 % skim milk for 1 h. Thereafter, primary and secondary antibodies were applied to the membranes for reaction. After washing with 1 × tris-buffered saline Tween (TBST) for 1 min, the chemiluminescent substrate kit was used for exposure of the protein band.

Evaluation of caspase-3 activity

Caspase-3 activity was determined using the relative commercial kit (Beyotime, Shanghai, China). Briefly, transfected cells were lysed and the protein levels were determined by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). The protein sample was incubated with acetyl-Asp-Glu-Val-Asp p-nitroanilide for 1 h, and absorbance was measured at 405 nm. Each experiment was performed in triplicate.

Statistical analysis

SPSS 20.0 (SPSS, Chicago, IL, USA) was used for data analysis. Data are expressed as mean ± standard deviation (SD). Kaplan-Meier method was applied for survival analysis. Comparison between two groups were made using t-test. P < 0.05 was considered statistically significant.

RESULTS

TUSC7 was downregulated in GC and predicted a poor prognosis

The expression level of TUSC7 was lower in GC tissues compared to adjacent normal tissues (Figure 1 A). TUSC7 was identically downregulated in GC cells (Figure 1 D). In particular, TUSC7 level was higher in GC with stage I+II relative to those with stage III+IV (Figure 1 B). Based on the median level of TUSC7 in enrolled GC patients, the patients were assigned into high-level and low-level TUSC7 groups. Kaplan-Meier curves revealed worse prognosis in GC patients of low-level TUSC7 group (Figure 1 C).

E2F6 was upregulated in GC

E2F6 was highly expressed in GC tissues and cell lines (Figure 1 E and G). GC patients with stage I+II expressed lower level of E2F6 compared with those of stage III+IV patients.
Spearman correlation test revealed that E2F6 level was negatively correlated with TUSC7 in GC tissues ($R = -0.5826$, $p < 0.001$; Figure 1 H).

**Figure 1:** Downregulation of TUSC7 and upregulation of E2F6 in GC. (A) TUSC7 levels in gastric cancer tissues and normal tissues. (B) TUSC7 levels in gastric cancer tissues with stage I+II or stage III+IV. (C) Overall survival in gastric cancer patients expressing high or low level of TUSC7. (D) TUSC7 levels in gastric mucosal cells (GES1) and gastric cancer cells (AGS, BGC823, SGC7901 and MGC803). (E) E2F6 levels in gastric cancer tissues and normal tissues. (F) E2F6 levels in gastric cancer tissues and normal tissues. (G) E2F6 levels in gastric mucosal cells (GES1) and gastric cancer cells (AGS, BGC823, SGC7901 and MGC803). (H) A negative correlation between expression levels of TUSC7 and E2F6 in gastric cancer tissues. All data were presented as mean ± SD; *$p < 0.05$ vs. NC group, **$p < 0.01$ vs. NC group

**Figure 2:** E2F6 negatively regulated TUSC7 level. (A) Transfection efficacy of LV-E2F6 in BGC823 and SGC7901 cells. (B) Transfection efficacy of si-E2F6 in BGC823 and SGC7901 cells. (C) TUSC7 level in BGC823 and SGC7901 cells transfected with LV-NC or LV-E2F6. (D) TUSC7 level in BGC823 and SGC7901 cells transfected with si-NC or si-E2F6. (E, F) Luciferase activity in BGC823 and SGC7901 cells. All data are presented as mean ± SD; **$p < 0.01$ vs. NC group, ***$p < 0.001$ vs. NC group

Knockdown of TUSC7 partially reversed the regulatory effects of E2F6 on GC

The results demonstrate that transfecting si-E2F6 led to a decrease in viability and invasiveness in BGC823 and SGC7901 cells. Interestingly, the reduced viability and invasiveness in GC cells with E2F6 knockdown were partially reversed by silence of TUSC7 (Figures 3 A and B). The apoptotic rate increased in GC cells transfected with si-E2F6, which was further reversed by co-transfection of si-TUSC7 (Figure 3 C). Hence, TUSC7 was responsible for E2F6-regulated phenotypes in GC cells.

**E2F6/TUSC7 regulated MMP-2 and caspase-3 in GC**

Protein level of MMP-2 was downregulated in BGC823 and SGC7901 cells transfected with si-E2F6, which was further elevated by co-transfection of si-TUSC7 (Figure 4 A). In addition, caspase-3 activity increased in GC cells with E2F6 knockdown, but it was reduced by co-transfection of si-TUSC7 (Figure 4 B). Thus, E2F6/TUSC7 may affect invasiveness and apoptosis in GC by regulating MMP-2 expression and caspase-3 activity.
Figure 3: Knockdown of TUSC7 partially reversed the regulatory effects of E2F6 on GC. BGC823 and SGC7901 cells were transfected with si-NC, si-E2F6 or si-E2F6+si-TUSC7. (A) Viability; (B) Invasion of cell number (magnification: 20×); (C) Apoptotic rate. *P < 0.05 vs. si-NC, **p < 0.01 vs. si-NC

Figure 4: E2F6/TUSC7 regulated MMP-2 and caspase-3 in GC. BGC823 and SGC7901 cells were transfected with si-NC, si-E2F6 or si-E2F6+si-TUSC7. (A) Protein level of MMP-2; (B) Caspase-3 activity. *P < 0.05 vs. si-NC, **p < 0.01 vs. si-NC

DISCUSSION

Gastric cancer (GC) is a common malignancy posing a great burden on affected people. Although the morbidity and mortality of GC have been reduced, the overall survival is still very low. Surgical procedures are the only treatment strategy for GC. However, most GC patients’ conditions have deteriorated and are at an advanced stage at initial diagnosis, and thus miss the optimal opportunity for surgery [13]. Chemotherapy, radiotherapy and targeted therapy show a relatively high response for advanced GC [14]. It is believed that early detection of GC is the most fundamental approach for improving the prognosis of GC. Tumor invasion and metastasis are the major reasons for the poor prognosis of GC patients, which greatly affect the effectiveness of GC treatment [15].

LncRNAs are differentially expressed in tumor tissues and normal tissues. Based on the specific functions, some lncRNAs are highly expressed in tumor tissues as oncogenes [16], while others are downregulated as tumor-suppressor genes.

The E2F transcription factor family is capable of regulating cell cycle, DNA replication-associated gene expressions, DNA repair, mitosis, and cell fate. Recent studies have shown the critical functions of E2F in cell apoptosis and senescence. E2F6 is one of the members of the E2F transcription factor family. It has a conserved DNA-binding region and a DP-polymerized region similar to other E2Fs members. However, E2F6 lacks a carboxy-terminal sequence that primarily functions as pocket protein binding and transcriptional activation. As a result, E2F6 is only able to exert an inhibitory effect on gene transcription through an Rb-independent manner [17]. It has been reported that E2F6 inhibits the transcription of E2F-dependent genes in the presence of the carboxy terminus. E2F6 has been shown to suppress the transcription of tumor-associated genes by inhibiting their promoters. Proteins encoded by them are of significance in tumor suppression and maintenance of chromosome structure [18].

In the present work, TUSC7 was downregulated in GC tissues, and its level was closely linked to tumor staging and prognosis of GC. Therefore, TUSC7 potentially influences the progression of GC. Conversely, E2F6 was upregulated in GC, and its level was negatively correlated to that of TUSC7. Dual-luciferase reporter gene assay results further confirmed binding between TUSC7 and E2F6.
CONCLUSION

TUSC7 expression is decreased in gastric cancer, and its low levels are associated with worse prognosis for patients with this disease. E2F6 plays a role in promoting the proliferation and invasion of gastric cancer cells by downregulating TUSC7. Targeting E2F6/TUSC7 axis may hold promise as a therapeutic approach for the management of gastric cancer.

DECLARATIONS

Acknowledgements

None provided.

Funding

None provided.

Ethical approval

None provided.

Availability of data and materials

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

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. Xingxing Huang and Jia Qiao contributed equally.

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doi: 10.4314/tjpr.v21i10.5