KLF7 Promotes Gastric Carcinogenesis Through Regulation of ANTXR1

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Purpose: Elucidating the mechanism of gastric cancer progression is of great importance for the discovery of new therapy targets against gastric cancer. In this study, we investigated the function of Kruppel-like factor 7 (KLF7) in gastric cancer.

Methods: qPCR and Western blot were performed to determine the expression of ANTXR1 after KLF7 inhibition. CCK-8, colony formation, apoptosis analysis, cell cycle analysis and transwell assay were performed to determine KLF7 functions in cellular proliferation, migration, apoptosis and cell cycle. Tumour xenograft experiments were performed to examine cell growth in vivo.

Results: The results showed that KLF7 was upregulated in gastric cancer. The proliferation and migration of gastric cancer cells were suppressed by depletion of KLF7. In vivo tumour progression was also attenuated following the downregulation of KLF7. Meanwhile, overexpression of KLF7 promoted the proliferation and migration of gastric cancer cells. The results of the mechanistic analysis showed that KLF7 promoted gastric carcinogenesis via upregulation of ANTXR cell adhesion molecule 1 (ANTXR1).

Conclusion: Therefore, this study may provide a theoretical foundation for further clinical therapy of gastric cancer.

Keywords: KLF7, gastric cancer, ANTXR1, proliferation, migration

Introduction
Gastric cancer (GC) is the most common malignancy of the digestive system, with the second-highest fatality rate worldwide.1,2 Owing to the lack of early screening for GC, most patients with GC are diagnosed at a late stage of the disease, resulting in a decreasing 5-year survival rate.3 In addition, GC is a multistep and multifactorial process, and its progression involves multiple genes and environmental factors.1,4,5 These facts render the therapeutic strategy complex and less effective. Multimodal therapies, including chemotherapy, radiotherapy, and more recently immunotherapy, are well established and can improve the survival of patients with GC.6 However, most GCs remain immedicable. Thus, understanding the molecular events critical for GC initiation and progression is of great importance for the discovery of valuable diagnostic biomarkers and development of effective therapeutic strategies.

Kruppel-like factors (KLFs), conserved among mammals from human to mouse,7 are transcriptional regulators belonging to the zinc-finger family.8 Diverse cellular processes (eg, cell proliferation, differentiation, adipogenesis, and metabolism) are regulated by KLFs.9–11 The functions of KLFs during the development of cancer have been widely investigated. KLF1, KLF3, KLF5, and
KLF8 exhibit oncogenic potentials in various types of cancer, whereas KLF2, KLF4, and KLF6 have demonstrated tumour-suppressing activity.12,13 KLF7, which is highly expressed in numerous human tissues, also plays critical roles in various types of cancer.14,15 In GC, KLF7 effectively destroys the histological barrier that blocks tumour formation, and eventually promotes GC progression.15,16 KLF7 is upregulated in pancreatic ductal adenocarcinoma and its inhibition blocks pancreatic ductal adenocarcinoma tumour growth and metastasis in cell culture and mice.17 Guan et al demonstrate that, in glioma, KLF7 promotes polyamine biosynthesis and glioma development through the transcriptional activation of argininosuccinate lyase.14 Collectively, these previous reports indicate that KLF7 may be an oncogene. Thus, better understanding of the mechanism of KLF7 in cancer may provide effective therapeutic strategies.

Anthrax toxin receptor 1 (ANTXR1), also named as tumor endothelial marker 8 (TEM8), is identified as a receptor for protective antigen of anthrax toxin and highly conserved transmembrane glycoprotein, overexpressed in several types of cancers: including gastric cancer, breast cancer, colon cancer and pancreatic tumors.18 Additionally, as previous studies suggested, ANTXR1 could interacted with collagen I and actin cytoskeleton through its extracellular and intracellular domain, respectively, and acted as an adhesion molecular for cell spreading.19 Recent studies demonstrated that ANTXR1 was overexpressed in gastric cancer tissues, and could be functioned as a promising molecular biomarker for clinical application and correlated with stromal and immune cell infiltration in gastric cancer.20,21

In the present study, we aimed to investigate the clinical significance, biological function and molecular mechanisms of KLF7 in GC. To this aim, we examined the expression of KLF7 in GC tissues and performed loss-of-function and gain-of-function experiments in GC cells. Our study demonstrated that KLF7 upregulation of ANTXR cell adhesion molecule 1 (ANTXR1) contributed to GC growth and migration.

**Materials and Methods**

**Patient Information**

In total, 18 patients with GC were enrolled between June and December 2019 in the Second Affiliated Hospital of Qiqihar Medical University (Qiqihar, China). Adjacent normal tissues were obtained from an area >2 cm distal to the primary neoplasms. The study was approved by the Ethics Committee of The Second Affiliated Hospital of Qiqihar Medical University. Written informed consent was provided by all patients, which was conducted in accordance with the Declaration of Helsinki.

**The Cancer Genome Atlas (TCGA) GC mRNA Database and Analysis**

Normalized transcriptome expression datasets for GC were downloaded from the TCGA via Genomic Data Commons Data Portal, using ENCORI Pan-Cancer Analysis Platform ([http://starbase.sysu.edu.cn/index.php](http://starbase.sysu.edu.cn/index.php)). A total of 407 samples, containing transcriptional expression data of 375 tumour tissues and 32 normal tissues, were available for KLF7 and ANTXR1 expression analysis. As for survival analysis, a total of 365 GC tissues were included in this study.

**Cell Culture and Transfection**

AGS and HGC27 cells, purchased from ATCC, were cultured in Ham’s F-12K medium (Gibco; Thermo Fisher Scientific, Inc.) or Dulbecco’s modified Eagle’s medium (DMEM)/F-12 medium (Gibco; Thermo Fisher Scientific) supplemented with 2 mM glutamine and 10% foetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). The cells were maintained at 37°C under a humidified atmosphere containing 5% CO₂.

The siRNAs of KLF7 and ANTXR1 were purchased from Sigma. Lipofectamine RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc.) was used for the transfection of siRNA according to the instructions provided by the manufacturer. Lipofectamine 3000 (Thermo Fisher Scientific, Inc.) was used for the overexpression of the plasmid.

**Cell Counting Kit-8 (CCK-8) Cell Viability Assay**

The CCK-8 reagent was purchased from Sigma. Cells were seeded into 96-well plates at a density of 2000 cells per well. After transfection for an indicated period, CCK-8 reagent (10 µL) was added to each well, and the cells were incubated at 37°C for 3 h. The absorbance at 450 nm was measured using a microplate reader.

**Colony Formation**

AGS and HGC27 cells were seeded into six-well plates at a density of 1000 cells, and the culture medium was
replaced every 3 days. The colonies were formed after culturing the cells for 14 days. After washing thrice with phosphate-buffered saline, colonies were fixed by 4% paraformaldehyde for 20 min and stained using GIEMSA staining solution for 20 min. Formations with more than 50 cells were identified as colonies.

RNA Extraction, Reverse Transcription, and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)
TRIzol (Thermo Fisher Scientific, Inc.) was used for the isolation of total RNA from cells or tissues. The RNA was reversely transcribed using M-MLV-RTase (Promega) following the instructions provided by the manufacturer. qRT-PCR analysis was performed using the SYBR Master Mixture (TAKARA) on the Agilent MX3000p Real time PCR system. The qRT-PCR primers were as follows: KLF7 forward, 5’-AGACATGCCTTGAATTTGG AACG-3’ and reverse, 5’-GGGTCTAAGCGACGGAA G-3’; ANTXR1 forward 5’-CGGATTGCCGGACAGTAA GAT-3 and reverse, 5’-TCCTCTCAAGACAACCTTGA ATG-3’; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward, 5’-TGACTTCAACAGCGACACCCA-3’, and reverse, 5’-CACCTGTGTGCTGTAAGCACA-3’.

Apoptosis Analysis
The eBioscience™ Annexin V-fluorescein isothiocyanate (Annexin V-FITC) apoptosis detection kit (Thermo Fisher Scientific, Inc.) was used for the determination of cell apoptosis. Cell suspensions were incubated with Annexin V-FITC (5 µL) for 10–15 min. After incubation, the cells were washed with 1× binding buffer and resuspended in 1× binding buffer. The resuspended cells were incubated with propidium iodide (20 µg/mL). Finally, samples were subjected to flow cytometry analysis.

Cell Cycle Analysis
FxCycle propidium iodide (PI)/RNase Staining Solution (Thermo Fisher Scientific, Inc.) was used to analyse the cell cycle according to the instructions provided by the manufacturer. Briefly, cells were trypsinized and centrifuged at 13,000 rpm for 5 min. After washing with iced D-Hanks (pH=7.2-7.4) buffer, cells were fixed with iced 75% ethanol for at least 1 h. Subsequently, the cells were centrifuged and washed by D-Hanks, followed by incubations with 0.5 mL of FxCycle™ PI/RNase staining solution for 15–30 min at room temperature. Finally, without washing, the samples were analysed using 488-nm excitation, and emission data were collected using a 585/42 bandpass filter.

Western Blotting Analysis
Cells were lysed with radioimmunoprecipitation assay buffer containing proteinase inhibitors. The protein concentration was measured using the Bradford assay (Sigma; Merck). Subsequently, total protein (20 µg) was added to each well of a 10% sodium dodecyl sulfate-polyacrylamide gel for electrophoresis. Next, the proteins were transferred to nitrocellulose membranes and blocked with 5% non-fat milk at room temperature for 1 h. The membranes were incubated with KLF7 (1:2000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), ANTXR1 (1:2000; Proteintech), and GAPDH (1:5000; Proteintech) at 4°C overnight, followed by incubation with a secondary antibody (Thermo Fisher Scientific) at room temperature for 60 min.

Transwell Assay
Cells (3.0×104 per well) were seeded into the upper chamber of 24-well Corning® FluoroBlok TM Cell Culture Inserts (Corning Inc., Corning, NY, USA) to detect cell migration. The lower chambers were filled with Ham’s F-12K or DMEM/F-12 medium supplemented with 10% foetal bovine serum, which served as a chemoattractant. Finally, cells that had migrated to the other side of the filter were stained with 0.5% crystal violet and counted under an inverted fluorescence microscope.

Tumour Xenograft Experiments
Firstly, stable MKN45 cells infected with the shCtrl or shKLF7-expressing lentiviruses (GenePharma, Suzhou, China) using a supernatant fluid. Then, the shCtrl or shKLF7 MKN45 cells (2×106) were subcutaneously injected into the enterocoelia of immunodeficient nude mice (4-week-old, female BALB/c). Tumour weight and volume were calculated according to the international criteria: V=ab² (a, the longest diameter; b, the shortest diameter). Mice study was approved by the Ethics Committee of The Second Affiliated Hospital of Qiqihar Medical University and performed according to the Guideline of the Care and Use of Laboratory Animals in Qiqihar Medical University.

Statistical Analysis
All statistical analyses were performed using the SPSS version 25.0 (IBM Corp., Armonk, NY, USA) software.
The data were presented as the mean ± standard error of the mean of at least three independent repeats. Student’s t-test was applied to evaluate the difference between two groups. Differences among groups were determined by one- or two-way analysis of variance with repeated measures, followed by the Bonferroni post-hoc test. P-values <0.05 denoted statistical significance.

Results
Upregulation of KLF7 in GC
Firstly, samples from TCGA database were analysed by ENCORI Pan-Cancer Analysis Platform to investigate the relationship between KLF7 and GC. The results showed that KLF7 was upregulated in stomach adenocarcinoma (STAD) compared with normal samples (Figure 1A). qRT-PCR was performed to confirm these findings, revealing higher KLF7 expression levels in GC samples (Figure 1B). The correlation observed between the probability of survival for patients with GC and KLF7 expression showed that lower KLF7 expression extended the survival period and increased the survival rate of patients with STAD (Figure 1C). The above data indicated that KLF7 may be an oncogene.

Downregulation of KLF7 Suppressed Proliferation and Migration
To further elucidate the role of KLF7 in GC, siRNA was used to downregulate the expression level of KLF7 in AGS, HGC27 and MKN45 GC cells. Western blotting and qRT-PCR confirmed the high knockdown efficiency of siKLF7 in both cells (Figure 2A–D and Figure S1A). Subsequently, cell proliferation was analysed through the CCK-8 assay. Depletion of KLF7 apparently suppressed the proliferation of AGS, HGC27 and MKN45 GC cells (Figure 2E, H and Figure S1B). At day 4, the viability percentage was nearly half of that recorded for control cells (Figure 2E, H and Figure S1B). The colony formation assay showed that fewer colonies were formed in siKLF7 cells, with the colony numbers being reduced by >50% (Figure 2F, G, I, and J). Meanwhile, the migratory ability of cells was also impaired. Following KLF7 depletion, the percentage of migrated cells decreased by 50% compared with that of the control cells (Figure 2K and L). Thus, KLF7 expression is critical for the function of GC cells.

Downregulation of KLF7 Promotes Apoptosis and Cell Cycle Arrest
Flow cytometry analysis was performed to investigate the reasons responsible for the impaired proliferation and migration upon KLF7 depletion. Compared with control cells, depletion of KLF7 significantly induced apoptosis (Figure 3A and B). In AGS cells, apoptosis occurred in nearly half of the total number of cells upon depletion of KLF7 (Figure 3A). For HGC27 cells, the percentage of apoptotic cells tripled in those transfected with siKLF7 compared with control (Figure 3B). Cell cycle progression analysis
Figure 2 Knockdown of KLF7 suppresses cell proliferation and migration. (A and B) Effective knockdown of KLF7 in AGS cells. qRT-PCR (A) and Western blotting (B) analysis of AGS cells transfected with siNC, siKLF7-1, and siKLF7-2. (C and D) Effective knockdown of KLF7 in HGC27 cells. qRT-PCR (C) and Western blotting (D) analysis of HGC27 cells transfected with siNC, siKLF7-1, and siKLF7-2. (E) AGS cells transfected with siNC, siKLF7-1, and siKLF7-2 were subjected to analysis of cell proliferation through the CCK-8 assay. Knockdown of KLF7 suppresses cell proliferation in AGS cells. (F and G) Colony formation assay of AGS cells transfected with siNC, siKLF7-1, and siKLF7-2. (H) HGC27 cells transfected with siNC, siKLF7-1, and siKLF7-2 were subjected to analysis of cell proliferation using the CCK-8 assay. Knockdown of KLF7 suppresses cell proliferation in HGC27 cells. (I and J) Colony formation assay of HGC27 cells transfected with siNC, siKLF7-1, and siKLF7-2. (K and L) Transwell invasion analysis of AGS and HGC27 cells transfected with siNC, siKLF7-1, and siKLF7-2; images were captured with a 40× magnification. **p<0.01, ***p<0.001.
showed that knockdown of KLF7 decreased the percentage of cells in the S and G2/M phases; in contrast, the percentage of cells in the G0 and G1 phases increased (Figure 3C and D). Collectively, this evidence shows that KLF7 regulates cell apoptosis and cell cycle progression.

**Overexpression of KLF7 Promoted Proliferation and Migration, and Inhibited Apoptosis**

As a potential oncogene, overexpression of KLF7 would promote tumour progression. Overexpression of KLF7 in both AGS and HGC27 cells was confirmed by Western
blotting and qRT-PCR (Figure 4A and B). Consistent with previous knockdown results (Figures 2 and 3), overexpression of KLF7 significantly promoted cell viability (Figure 4C and D). The ability for colony formation was also enhanced; the number of colonies nearly tripled in exogenous KLF7 expression samples (Figure 4E and F). Apoptosis and migration were also altered following overexpression of KLF7. Flow cytometry analysis showed an approximately 50% reduction of apoptosis in KLF7-overexpressing cells compared with controls (Figure 4G and H). The proportion of migrated cells was elevated by three-fold after overexpression of KLF7 (Figure 4I and J). Taken together, the above data indicate that KLF7 is an oncogene.

Downregulation of KLF7 Suppressed Tumour Growth

To further clarify the role of KLF7 in tumour progression, tumour xenograft experiments were performed in vivo. shRNA targeting KLF7 was used to downregulate the
expression of KLF7. The tumour was isolated and measured at indicated time points (Figure 5A). Tumour volumes were significantly reduced following KLF7 depletion to only approximately 30% of the control volumes at day 36 (Figure 5B). Meanwhile, the results of the statistical analysis showed that the tumour weight decreased in shKLF7 samples (Figure 5B). The weight of control samples and shKLF7 tumours was 0.4 g and 0.1 g, respectively (Figure 5C). These results confirmed the oncogenic role of KLF7 in vivo.

KLF7 Regulated Cell Proliferation and Migration Through ANTXR1
The correlation between KLF7 and ANTXR1 was analysed to further clarify the mechanism involved in this process. Initially, we searched TCGA database and found a positive correlation between KLF7 and ANTXR1 (Figure 6A). Higher ANTXR1 expression shortened the overall survival period and decreased the survival percentage of patients with STAD (Figure 6B). These findings were consistent with those for KLF7 (Figure 1). Next, we investigated the effect of KLF7 on the expression of ANTXR1. Following KLF7 depletion, the expression of ANTXR1 was reduced at both the mRNA and protein levels in vitro and in vivo studies, respectively (Figure 6C–D and 5D). In addition, overexpression of KLF7 significantly upregulated the expression of ANTXR1 (Figure 6E and F).

To further investigate the relationship between these two proteins, we transfected cells with KLF7 plasmids and ANTXR1 siRNA. Western blotting showed that knockdown of ANTXR1 counteracted the upregulation of ANTXR1 induced by overexpression of KLF7 (Figure 6G). The higher cell viability observed after overexpression of KLF7 was reduced to the control level in KLF7+siANTXR1 samples (Figure 6H). The processes of colony formation and migration enhanced by KLF7 were also reversed following depletion of ANTXR1 (Figure 6I–L). Therefore, KLF7 and ANTXR1 played a synergistic role in gastric tumour progression.

Discussion
Gastric carcinoma is characterized by high mortality rates, difficulty for early diagnosis, and poor prognosis, representing a major health burden. Therefore, it is of great importance to elucidate the molecular mechanisms of GC progression for the development of effective diagnostic methods and therapies.

Figure 5 Downregulation of KLF7 suppresses xenografted tumour growth. (A) MKN45 cell lines expressing shCtrl or shKLF7 were injected into athymic nude mice (n=5) and analysed for tumour formation. Representative images of xenografted tumours formed with shCtrl or shKLF7 MKN45 cells (2×10⁶ cells/mouse). (B) The average tumour volumes at the indicated time points. (C) Quantification of the weight of tumours formed with shCtrl or shKLF7 MKN45 cells (2×10⁶ cells/mouse). ***p<0.001, **p<0.01. (D) Western blot was performed to determine the expression levels of KLF7 and ANTXR1 in xenografted tumours after KLF7 knockdown.
In this study, we focused on the role of KLF7 and ANTXR1 in GC. Firstly, KLF7 was highly correlated with GC progression. Downregulation of KLF7 promoted apoptosis and arrested the cell cycle, which in turn suppressed cell proliferation and migration (Figures 2–4). Next, it was shown that KLF7 acts as an oncogene. It is highly expressed in GCs (Figure 1A) and depletion of KLF7 inhibited tumour growth (Figure 5). Nevertheless, only 18 paired GC tissues were performed to determine KLF7 expression, which might be a limitation in this study.

Figure 6 KLF7 regulates cancer proliferation and migration through ANTXR1. (A) Correlation between KLF7 and ANTXR1 mRNA expression in stomach adenocarcinoma tissues (n=375) from TCGA database was analysed using the StarBase v2.0 database. (B) Correlation between the probability of survival for patients with gastric cancer and ANTXR1 expression. (C and D) qRT-PCR and Western blotting analysis of ANTXR1 expression in cells transfected with siCtrl and siKLF7. ANTXR1 was downregulated in cells transfected with siKLF7. For qRT-PCR, the expression levels were normalized to those of β-actin. For Western blotting, GAPDH was used as a loading control. (E and F) ANTXR1 was upregulated upon the overexpression of KLF7. qRT-PCR and Western blotting analysis of ANTXR1 expression in Ctrl and KLF7-overexpressing cells. For qRT-PCR, the expression levels were normalized to those of β-actin. For Western blotting, GAPDH was used as a loading control. (G) Western blotting analysis of AGS cells transfected with control, KLF7, or KLF7+siANTXR1. GAPDH was used as a loading control. (H) CCK-8 cell viability assay of AGS cells transfected with control, KLF7 or KLF7+siANTXR1. (I and J) Transwell invasion analysis of indicated AGS cells. (K and L) Colony formation assay of indicated AGS cells. ***p<0.001, **p<0.01.

Abbreviation: ns, not significant.
study. While, the expression of KLF7 in gastric cancer tissues have been analyzed in 375 stomach adenocarcinoma samples, which could be further confirmed that KLF7 was upregulated in gastric cancer tissues.

Thus, KLF7 may act in a similar manner to its homologous proteins KLF1, KLF3, KLF5, and KLF8. Thirdly, KLF7 functions in GC through regulation of ANTXR1, a known potential target in diverse types of cancer. This was the first study investigating the role of the KLF7-ANTXR1 axis in the progression of GC. KLFs are transcriptional regulators of the zinc-finger family, which participate in numerous essential biological cellular processes, such as proliferation, differentiation, apoptosis, and migration. In the last decades, more attention has been drawn to the role of KLFs in cancer. For example, KLF5 promotes cell migration in bladder cancer cells, while KLF8 enhances breast cancer cell invasion and metastasis. However, in hepatocellular carcinoma, KLF4 functions as a tumour suppressor. Therefore, different KLFs play various roles in different cancers, and their functions are synergistic or antagonistic. In GC, KLF7 plays an oncogenic role. However, the function of other KLFs in relation to KLF7 warrants further investigation.

As previously reported, KLFs regulate gene expression by recognizing the GC-rich sequences in the proximal promoters of target genes. Our study demonstrated that KLF7 regulated cancer cell proliferation and migration through ANTXR1 (Figure 6). Nevertheless, whether KLF7 regulates ANTXR1 through binding to its promoter remains to be determined. Also, the number of GC-rich sites present in the promoter of ANTXR1, and the site to which KLF7 binds require further investigation. These points warrant clarification to pave the way for understanding the mechanism of the KLF7-ANTXR1 axis.

The ultimate goal of cancer research is to discover new targets for therapy. Several promising immunotherapy drugs have been approved by the US Food and Drug Administration for the treatment of locally advanced or metastatic GC, including trastuzumab (targeting ERBB2; human epidermal growth factor receptor 2), ramucirumab (targeting kinase insert domain receptor [KDR]; vascular endothelial growth factor receptor 2 [VEGFR2]), and pembrolizumab (targeting programmed cell death 1 [PD1]). However, additional immunotherapy drugs targeting different factors are needed to overcome GC. ANTXR1 is overexpressed in several types of cancer. Moreover, in a preclinical setting, an anti-ANTXR1 antibody-drug conjugate demonstrated high efficiency for augmenting therapies against diverse types of cancer. Thus, ANTXR1 has great potential to become an effective target for the treatment of GC. Further in vivo experiments are required to evaluate new potential drugs and demonstrate their effectiveness.

Conclusions
In summary, we provided the evidence that KLF7/ANTXR1 axis promoted gastric cancer cell growth and proliferation. The findings of the present study may help discover new immunotherapy drug for the treatment of GC.

Author Contributions
All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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Disclosure
These authors report no conflicts of interest in this work.

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