lncRNA LIFR-AS1 inhibits gastric carcinoma cell proliferation, migration and invasion by sponging miR-4698

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Abstract. The vital functions of long non-coding (lnc)RNAs have been verified in gastric carcinoma (GC). However, as a novel cancer-related lncRNA, the influence of leukemia inhibitory factor receptor antisense RNA 1 (LIFR-AS1) in GC cell biological behaviors remains unreported. The present study explored the biological effects of lncRNA LIFR-AS1 on GC progression. Reverse transcription-quantitative PCR was performed to examine lncRNA LIFR-AS1 expression in GC tissues and cells. Cell Counting Kit-8, 5-ethynyl-2'-deoxyuridine incorporation, cell wound healing and Transwell invasion assays were used to assess the functions of lncRNA LIFR-AS1 in GC cell proliferation, migration and invasion. Additionally, associations among lncRNA LIFR-AS1, microRNA (miR)-4698 and microtubule-associated tumor suppressor 1 (MTUS1) were investigated via bioinformatics software and a luciferase reporter system. In addition, western blotting was used to examine the expression of MEK and ERK. Decreased lncRNA LIFR-AS1 expression was observed in GC tissues and cells. Upregulated lncRNA LIFR-AS1 inhibited GC cell proliferation, migration and invasion. Upregulated miR-4698 and downregulated MTUS1 were identified in GC tissues and cells. The inhibitory interaction between lncRNA LIFR-AS1 and miR-4698 was confirmed. Additionally, MTUS1 was predicted as a target gene of miR-4698 positively regulated by lncRNA LIFR-AS1. The MEK/ERK pathway was inhibited by lncRNA LIFR-AS1 via regulating MTUS1. These findings revealed the inhibitory functions of lncRNA LIFR-AS1 in GC cell proliferation, migration and invasion. The process was mediated via miR-4698, MTUS1 and the MEK/ERK pathway.

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Introduction

Gastric carcinoma (GC) is a malignant tumor originating from the gastric mucosa (1). The incidence rate of GC is second only to lung carcinoma and is significantly higher in men than in women (2). Due to the GC tumor invasion and metastasis, GC affects the liver, kidney and respiratory functions and endangers life (3). The majority of patients with early GC generally have no distinct symptoms. However, with the growth of tumor, obvious symptoms appear as the gastric function is affected (4). In clinical practice, surgical treatment, chemotherapy, traditional Chinese medicine treatment and other comprehensive therapies are used to ameliorate the symptoms of patients with GC and prolong the survival (5,6). Nevertheless, the prognosis of patients with GC in advanced-stage is still unsatisfactory. Thus, it is important to investigated a tumor marker and develop a new curative method for GC.

Long non-coding (lnc)RNAs are transcripts >200 nucleotides in length (7). Increasing evidence has uncovered significant functions of lncRNAs in various types of cancer (8,9). Notably, a number of GC-associated lncRNAs such as H19 (10), HOXA11-AS (11) and AK058003 (12) have been reported to modulate the proliferative, migratory and invasive capacities of GC cells. The cancer-related lncRNA LIFR-AS1 has been studied in breast (13,14) and colorectal cancer (15). These studies have shown that lncRNA LIFR-AS1 is linked to the high survival rate of breast cancer and can inhibit breast cancer cell proliferation and migration; in addition, it can regulate the resistance of colorectal cancer to photodynamic therapy (14,15). However, the functions of lncRNA LIFR-AS1 in GC remain to be elucidated.

Micro (mi)RNAs are encoded by endogenous genes with a length of ~22 nucleotides and possess multiple vital regulating effects in a variety of cells (16,17). Accumulating evidence has established that lncRNAs work as miRNAs sponge to serve functions in the progression of various types of cancer, including GC (18,19). miR-4698 is situated on chromosome 12, which is linked to metastatic melanoma (20). Notably, Kalhori *et al* (21) revealed that miR-4698 can stop glioblastoma cell proliferation by controlling the PI3K/AKT pathway (21). Whether miR-4698 takes part in mediating the functions of lncRNA LIFR-AS1 in GC cells is the focus of the present study.

Microtubule-associated tumor suppressor 1 (MTUS1) is a tumor suppressor gene at chromosome 8p21.3-22, encoding a mitochondrial protein and controlling cellular proliferation (22). Previous studies have demonstrated that the expression status of MTUS1 is altered in several types of tumors, such as fibroadenoma and breast cancer (23), colorectal carcinoma (24) and bladder cancer (25). Thus far, no study has investigated whether MTUS1 participates in mediating the functions of lncRNA LIFR-AS1 in GC cells.

According to the above research, the present study preliminarily investigated the functions of lncRNA LIFR-AS1 in GC cell proliferation, migration and invasion and determined the molecular mechanisms involved. The results from the present study might offer a new research orientation for GC treatment from the aspect of lncRNA, miRNA and mRNA axis.

Materials and methods

Collection of samples from patients with GC. The GC tissues and paired adjacent tissues needed for the experiments were collected from 41 patients with GC (Table I). No treatment was performed on these patients prior to surgery. Patients were excluded if they had other clinical disorders. The obtained samples were frozen in liquid nitrogen and stored at -80˚C. All patients personally signed an informed consent. The protocol of the present study was approved by the Ethics Committee of of Cangzhou People's Hospital (approval number AF/SC-08/02.0).

Cell culture. GC cell lines (MKN45 and AGS) and the gastric mucosal epithelial cell line (GES-1) were obtained from the Cell Bank of the Chinese Academy of Sciences. RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.) was used to culture these cells. The culture conditions were 5% CO_2 and 95% air at 37˚C.

Cell transfection. The vectors of pcDNA-LIFR-AST and paired pcDNA negative control (NC), as well as miR-4698 mimics, miR-4698 inhibitors and their corresponding controls (mimics NC and inhibitors NC) used in this study were synthesized by GenePharma. The small interfering (si) RNA targeting microtubule-associated tumor suppressor 1 (MTUS1) was constructed in U6/GFP/Neo vectors (GenePharma) to silence MTUS1 expression. A scrambled siRNA was included as NC. The MKN45 and AGS cells were pre-incubated on 6-well plates until they reached ~60% confluence and the transfection was conducted with Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). A total of 50 nM pcDNA-LIFR-AST or pcDNA NC, 20 nM miR-4698 mimics or mimics NC, 20 nM miR-4698 inhibitors or inhibitors NC and 45 nM si-MTUS1 or si-NC were used. Cells were harvested 48 h post-transfection for further studies.

Detection of cell proliferation. When cell transfection was concluded, Cell Counting Kit-8 (CCK-8) and 5-ethynyl-2'-deoxyuridine (EdU) incorporation assays (Abcam) were used to assess cell proliferation. Transfected MKN45 and AGS cells ($5x10^3$ cells/well) were cultivated at 37˚C for 1, 2, 3 and 4 days. CCK-8 solution (10 μl) was added into each well for another 2 h incubation. A microplate reader (BioTek Instruments, Inc.) was used for examining the absorbance at 450 nm wavelength. For the EdU assay, transfected cells were grown in 24-well plates at 37˚C. The cells were then stained with 40 μM EdU solution at 37˚C for 2 h and then 4% formaldehyde and 10X Triton X-100 buffer (Sigma-Aldrich; Merck KGaA) were added at room temperature for 15 min incubation. The reaction mix was added to fluorescently labelled EdU and was incubated at 37˚C for 30 min. Finally, the EdU positive cells were analyzed by a fluorescence microscope (Olympus Corporation, magnification, x100). Images were captured in five random fields.

Wound healing assay. The transfected MKN45 and AGS cells were cultured in 6-well plates to produce a cell monolayer (100% confluence). The cell wounds were created using a 100 μl pipette tip. After washing three times with sterile PBS, cells were further cultured at 37˚C for 24 h and then the width of scratch was observed with an inverted microscope (Olympus Corporation, magnification, x100). Additionally, images were captured at 0 and 24 h after scratching. ImageJ software (v1.8.0.112, National Institutes of Health) was used to analyze the images. Images were captured in five random fields.

Cell invasion. The transfected MKN45 and AGS cells in 200 μl serum-free RPMI-1640 medium were added to the upper Transwell chambers (Corning Inc.; 24-well insert, pore size 8 mm). The Transwell membrane was pre-coated with Matrigel (BD Biosciences). Meanwhile, the bottom Transwell chambers were filled with 600 μl RPMI-1640 medium supplemented with 10% FBS. After 24 h incubation at 37˚C, the cells were fixed and subsequently stained with 0.5% crystal violet solution (Sigma-Aldrich; Merck KGaA) for 15 min at room temperature. The invasive cells were counted under a light microscope (Olympus Corporation, magnification, x100). ImageJ software (v1.8.0.112, National Institutes of Health) was used to analyze the images. Images were captured in five random fields.

Luciferase reporter assay. The lncRNA LIFR-AS1 fragment or the 3'-untranslated region (UTR) of MTUS1 was amplified by performing PCR. Then, the pmirGLO luciferase vector (Promega Corporation) was used to establish LIFR-AS1 wild type (WT) and MTUS1-WT. A site-directed mutagenesis kit (Thermo Fisher Scientific, Inc.) was employed to generate LIFR-AS1 mutant (MUT) or MTUS1-MUT. All the above constructed vectors and miR-4698 mimics (500 ng) were co-transfected into MKN45 and AGS cells using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). After 48 h transfection, the relative luciferase activity was assessed using a dual luciferase reporter assay system (Promega Corporation). *Renilla* luciferase activity was used for normalization.

Reverse transcription-quantitative (RT-q) PCR. Total RNA was isolated from GC tissues and cell lines ($5x10^6$ cells/ml) using TRIzol® reagent (Thermo Fisher

Table I. Correlation between LRFR-AS1 expression and clinical characteristics.

Variable	N (%)	Relative LRFR-AS1 expression ($2^{-\Delta\Delta Cq}$ method)	P-value
Age (year)	59.2±10.5	/	
Sex			0.2584
Male	27 (65.8%)	0.466±0.185	
Female	14 (34.2%)	0.541±0.223	
Histologic type			0.2711
Adenocarcinoma	35 (85.4%)	0.477±0.187	
Others	6 (14.6%)	0.575±0.264	
Differentiation degree			0.0383[a]
Well/Moderately	18 (43.9%)	0.564±0.209	
Poorly	23 (56.1%)	0.435±0.176	
Depth of invasion			0.1190
T1 and T2	16 (39.0%)	0.552±0.215	
T3 and T4	25 (61.0%)	0.452±0.182	
Nodal status			0.0045[b]
pN0	18 (43.9%)	0.588±0.184	
pN1-3	23 (56.1%)	0.416±0.180	
TNM stage			0.0011[b]
I, II	26 (63.4%)	0.565±0.187	
III, IV	15 (36.6%)	0.364±0.153	

[a]P<0.05, [b]P<0.01. TNM, TNM Classification of Malignant Tumors.

Scientific, Inc.) according to the manufacturer's protocols. A Reverse Transcription kit (Promega Corporation) was used to synthesize cDNA according to the manufacturer's protocols. SYBR® Green PCR Kit (Qiagen, Inc.) was used to observe lncRNA LIFR-AS1 and MTUS1 expression. All reactions were performed in a 10 μl reaction volume in triplicate. PCR were with conditions of 95˚C for 50 sec, followed by 40 cycles of 95˚C for 12 sec and 55.5˚C for 30 sec. For detecting miR-4698 expression, TaqMan Universal Master Mix II (Takara Biotechnology Co., Ltd.) was employed for RT-qPCR procedure. β-actin and U6 served as internal control for standardization of the data. All the data were analyzed by the $2^{-\Delta\Delta Cq}$ method (26). Primer sequences are listed in Table II.

Western blot assay. Following cell transfection, proteins were extracted with RIPA buffer with phenylmethanesulfonyl fluoride (Beyotime Institute of Biotechnology). The protein content was assessed using the BCA method. Afterwards, total protein (30 μg) was separated via 10% SDS-PAGE (Beyotime Institute of Biotechnology). The separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes (EMD Millipore). Following blocking with 5% skimmed milk powder for 2 h at room temperature, the primary antibodies of MTUS1 (1:1,000, cat. no. sc-393120, Santa Cruz Biotechnology Inc.), MEK (1:1,000, cat. no. ab32091), phosphorylated (p-)MEK (1:1,000, cat. no. ab96379), ERK (1:1,000, cat. no. ab184699), p-ERK (1:1,000, cat. no. ab201015), Cell division cycle-25 (Cdc25)B (1:1,000, cat. no. ab124819), cyclin-dependent kinase (Cdk)1 (1:1,000, cat. no. ab133327), p-Cdk1 (1:1,000, cat. no. ab201008) and GAPDH (1:2,000, cat. no. ab8245; all from Abcam) were incubated with the PVDF membranes overnight at 4˚C. Next, the appropriate secondary antibodies [1:5,000; goat anti-mouse IgG H&L (HRP) cat. no. ab205719 or goat anti-rabbit IgG H&L (HRP) cat. no. ab6721, Abcam] were used to incubate the above membranes for additional 1 h at room temperature. The protein levels were monitored by enhanced chemiluminescence reagents (Amersham Biosciences; Cytiva). The protein bands were quantified using Quantity One software (v4.6.6, Bio-Rad Laboratories, Inc.).

Bioinformatics analysis. The expression levels of lncRNA LIFR-AS1 in GC tumors and normal tissues was downloaded from The Cancer Genome Atlas (TCGA; https://portal.gdc.cancer.gov/). DIANA tools (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php) was used to predict potential binding miRNAs for lncRNA LIFR-AS1. The potential target gene of miRNA was predicted by TargetScan software, version 7.2 (http://www.targetscan. org/vert_72/).

Statistical analysis. All data are presented as the mean ± standard deviation. SPSS 19.0 statistical software (IBM Corp.) was used for statistical analyses. Relevance between lncRNA LIFR-AS1 and miR-4698 or MTUS1 was assessed by using Pearson correlation analysis. Comparisons between different groups were analyzed by using Student's t-test or one-way ANOVA followed by Tukey's multiple comparisons test. P<0.05 was considered to indicate a statistically significant difference.

Table II. Primers used for reverse transcription-quantitative PCR.

Primer name	Sequence
LIFR-AS1	Forward 5'-GCAAATACTGTGTATTAGTCC-3'
	Reverse 5'-CCGCTTCCTTGTGAAGAAGGT-3'
miR-4698	Forward 5'-TGGTACTGATGTGATGGACT-3'
	Reverse 5'-TCATATCACACAGCACCGAT-3'
MTUS1	Forward 5'-GAGCTGAGCACTTACAGCAACAA-3'
	Reverse 5'-TTCAACTGCATTAAGAGCTGTAA-3'
U6	Forward 5'-CTCGCTTCGGCAGCACA-3'
	Reverse 5'-AACGCTTCACGAATTTGCGT-3'
β-actin	Forward 5'-CCTGACGCCAACACACTGC-3'
	Reverse 5'-ATACTCCTGCTTGGTGATCC-3'

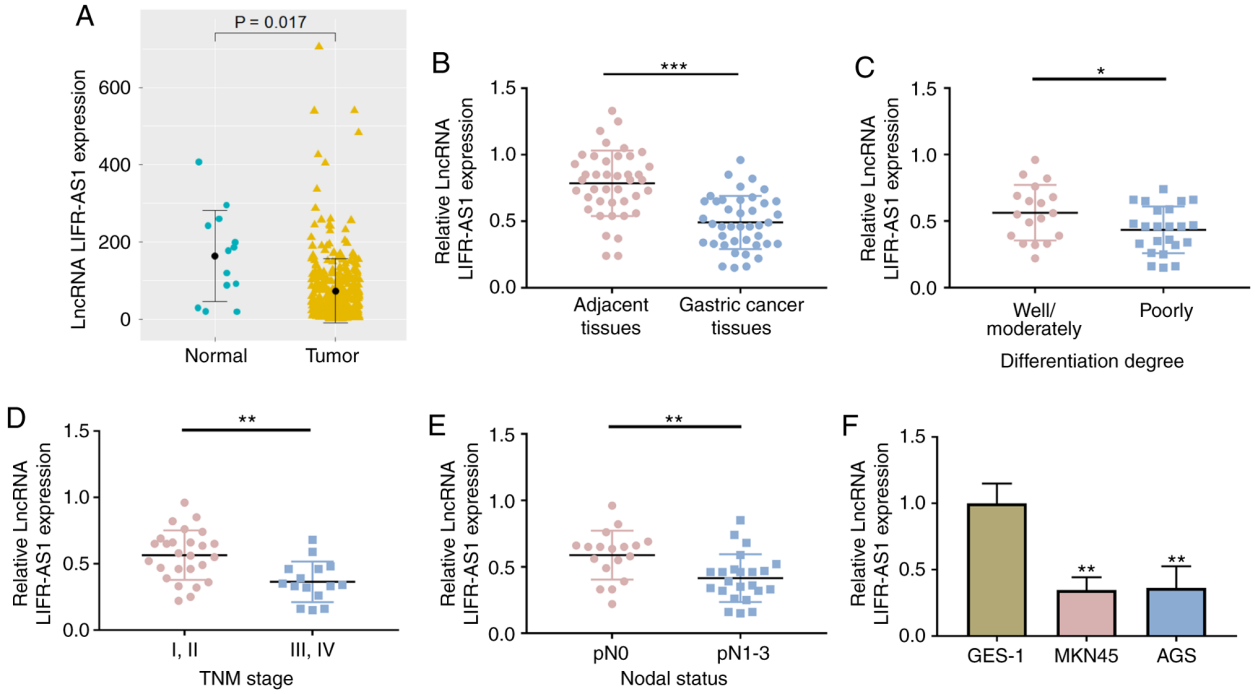

Figure 1. Decreased lncRNA LIFR-AS1 expression in GC tissues and cell lines. (A) Expression of lncRNA LIFR-AS1 in GC tissues and normal tissues were downloaded from the TCGA database. (B) Expression of lncRNA LIFR-AS1 in 41 pairs of GC tissues and matched adjacent tissues was determined using RT-qPCR. Expression of lncRNA LIFR-AS1 in 41 patients with GC with the condition of (C) well/moderately and poorly differentiation, (D) different TNM stages and (E) pN0-3 nodal status was investigated using RT-qPCR. (F) Expression of lncRNA LIFR-AS1 in GES-1 and GC (MKN45 and AGS) cell lines was assayed using RT-qPCR. *P<0.05, **P<0.01, ***P<0.001. lnc, long non-coding; GC, gastric carcinoma; TCGA, The Cancer Genome Atlas; RT-qPCR, reverse transcription-quantitative PCR; GES-1, gastric mucosal epithelial cell line.

Results

Downregulation of lncRNA LIFR-AS1 in GC tissues and cell lines. To investigate the biological role of LIFR-AS1 in GC, data from normal and tumor samples were obtained from TCGA and it was found that LIFR-AS1 was significantly downregulated in GC tissues compared with normal tissues (P=0.017; Fig. 1A). Expression of lncRNA LIFR-AS1 was examined in 41 GC tissues and the adjacent tissues using RT-qPCR. The low expression of lncRNA LIFR-AS1 was clearly presented in GC tissues (P<0.001, Fig. 1B). The relationship between lncRNA LIFR-AS1 expression and clinical characteristics is shown in Table I. In the GC tissues of patients with poorly differentiation degree, III and IV TNM stage and pN1-3 nodal status, the expression of lncRNA LIFR-AS1 was significantly reduced (P<0.05, P<0.01; Fig. 1C-E). Furthermore, the expression of lncRNA LIFR-AS1 was also explored in GES-1 and GC cell lines (MKN45 and AGS). It was identified that expression of lncRNA LIFR-AS1 was lower in MKN45 and AGS cells compared with that in GES-1 cells (P<0.01; Fig. 1F). These results demonstrated that lncRNA LIFR-AS1 was downregulated in GC tissues and cells.

Overexpression of lncRNA LIFR-AS1 restrains GC cell proliferation and movement. The overexpressed vector of lncRNA LIFR-AS1 (pcDNA-LIFR-AS1) was transfected into

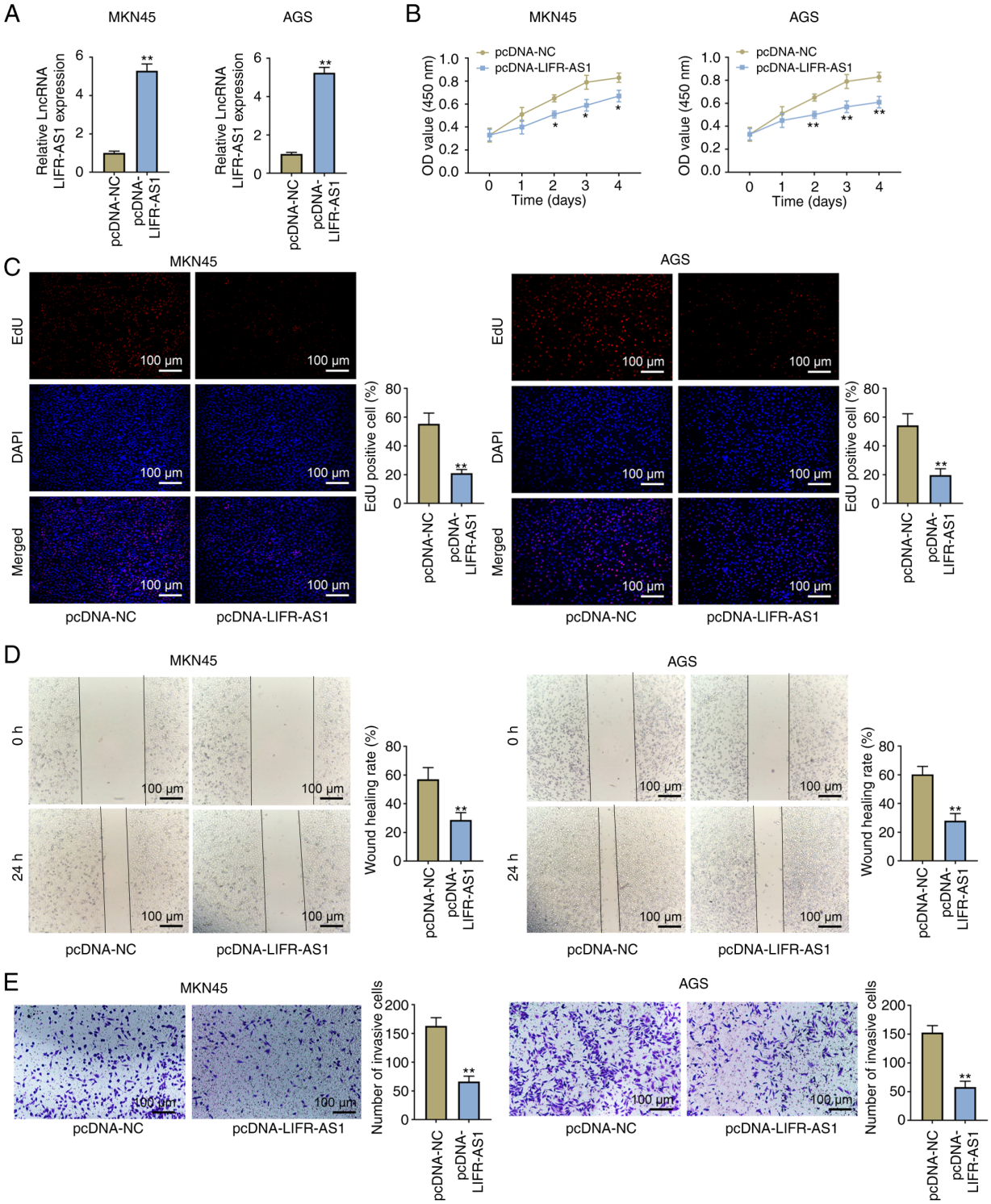

Figure 2. Overexpressed lncRNA LIFR-AS1 inhibits GC cell proliferation and migration. (A) Expression of lncRNA LIFR-AS1 in pcDNA-LIFR-AS1 and pcDNA-NC transfected GC (MKN45 and AGS) cell lines was explored using reverse transcription-quantitative PCR. After successful transfection, the proliferative abilities of MKN45 and AGS cells were assessed by (B) CCK-8 and (C) EdU; the migration capacities of MKN45 and AGS cells were evaluated using (D) wound healing and (E) Transwell invasion assays. Scale bar=100 μm. *P<0.05, **P<0.01 vs. pcDNA-NC group. lnc, long non-coding; GC, gastric carcinoma; NC, negative control; EdU, 5-ethynyl-2'-deoxyuridine.

MKN45 and AGS cells. Expression of lncRNA LIFR-AS1 was distinctly upregulated in pcDNA-LIFR-AS1-transfected MKN45 and AGS cells (P<0.01; Fig. 2A). CCK-8 experimental results revealed that overexpressed lncRNA LIFR-AS1 reduced cell viability at days 2, 3 and 4 in MKN45 and AGS cells (P<0.05, P<0.01; Fig. 2B). In addition, the number of EdU positive cells was also reduced by overexpressed lncRNA LIFR-AS1 (P<0.01; Fig. 2C). Cell migratory and invasive ability was then evaluated by wound and cell invasion assays. It was noted that overexpressed lncRNA LIFR-AS1 significantly decreased the wound healing areas in MKN45 and AGS cells (P<0.01; Fig. 2D). Similary, the capacity of cell invasion was

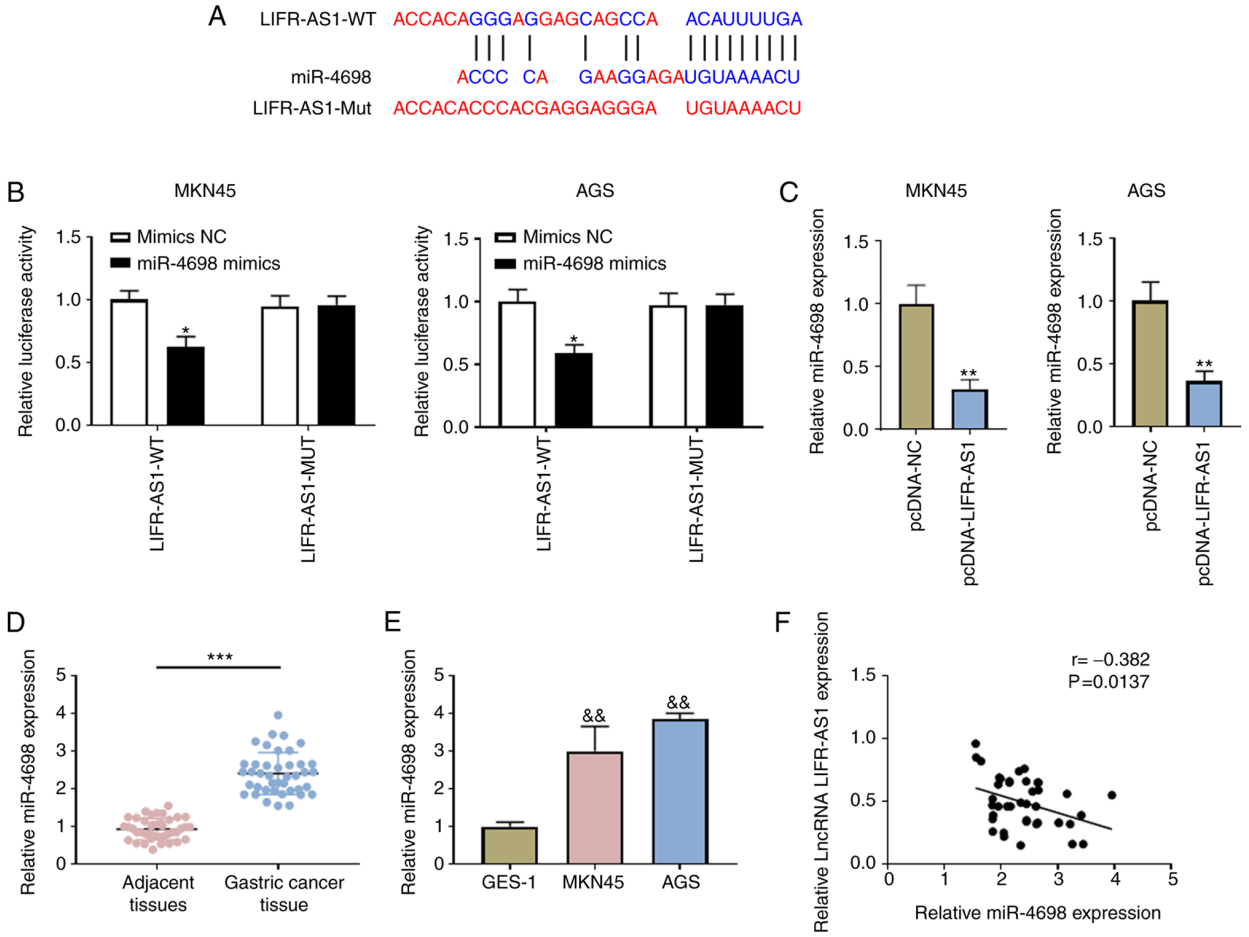

Figure 3. lncRNA LIFR-AS1 acts as a sponge for miR-4698. (A) The sequence of binding site between lncRNA LIFR-AS1 and miR-4698 was predicated using the online tool DIANA. (B) Association between lncRNA LIFR-AS1 and miR-4698 was investigated using a luciferase reporter system. (C) Expression of miR-4698 was established following pcDNA-LIFR-AS1 and pcDNA-NC transfection using RT-qPCR. Expression of miR-4698 in (D) 41 pairs of GC tissues and matched adjacent tissues and (E) in GES-1 and GC (MKN45 and AGS) cell lines was evaluated using RT-qPCR. (F) Mutual regulation between lncRNA LIFR-AS1 and miR-4698 was assessed using Pearson correlation analysis. *P<0.05 LIFR-AS1-WT+Mimics NC group, **P<0.01 vs. pcDNA-NC group, $^{\&\&}$P<0.01 vs. GES-1 group, ***P<0.001 vs. adjacent tissues group. lnc., long non-coding; miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR; GC, gastric carcinoma; GES-1, gastric mucosal epithelial cell line; Wt, wild type; Mut, mutant; NC, negative control.

also inhibited by overexpressed lncRNA LIFR-AS1 (P<0.01; Fig. 2E). All above results suggested that lncRNA LIFR-AS1 suppressed GC cell proliferation, migration and invasion.

Negative correlation between lncRNA LIFR-AS1 and miR-4698. Fig. 3A shows the predicted binding site of miR-4698 in lncRNA LIFR-AS1. To verify the predicted sequence binding sites between lncRNA LIFR-AS1 and miR-4698, luciferase reporter gene assay was performed to further confirm their association. It was observed that the luciferase activity was reduced in MKN45 and AGS cells with LIFR-AS1-WT and miR-4698 mimics co-transfection (P<0.05; Fig. 3B). It was noted that overexpressed lncRNA LIFR-AS1 decreased miR-4698 expression in MKN45 and AGS cells (P<0.01; Fig. 3C). Notably, upregulated miR-4698 expression was found in GC tissues (P<0.001; Fig. 3D) in addition to MKN45 and AGS cells (P<0.01; Fig. 3E). A Pearson correlation test revealed a negative correlation between lncRNA LIFR-AS1 and miR-4698 (P<0.05; Fig. 3F). All these findings demonstrated the negative correlation between lncRNA LIFR-AS1 and miR-4698 and that lncRNA LIFR-AS1 worked as a sponge of miR-4698 in GC cells.

MTUS1 is a target of miR-4698 and is positively associated with lncRNA LIFR-AS1. TargetScan revealed that the 3'-UTR of MTUS1 contained the complementary site for the seed region of miR-4698 (Fig. 4A). Following co-transfection with miR-4698 mimics and the fragments of MTUS1-WT or MTUS1-MUT, the change in the luciferase activity was evaluated. The results demonstrated inhibited luciferase activity in miR-4698 mimics and MTUS1-WT co-transfected cells (P<0.05; Fig. 4B). The overexpressed and suppressed vectors of miR-4698 (miR-4698 mimics and miR-4698 inhibitors) were transfected into GC cells and the correlation between miR-4698 and MTUS1 was monitored. Expression of miR-4698 was clearly enhanced in miR-4698 mimics-transfected cells (P<0.01), but restricted in miR-4698 inhibitors-transfected cells (P<0.05; Fig. 4C). Western blotting results revealed that MTUS1 expression was inhibited by miR-4698 overexpression and increased by miR-4698 inhibition (P<0.05; Fig. 4D). Fig. 4E and F demonstrate the downregulation of MTUS1 in GC tissues (P<0.001) and in GC (MKN45 and AGS) cell lines (P<0.01). A Pearson correlation test further confirmed the negative correlation between MTUS1 and miR-4698 (Fig. 4G) and the positive correlation between MTUS1 and lncRNA

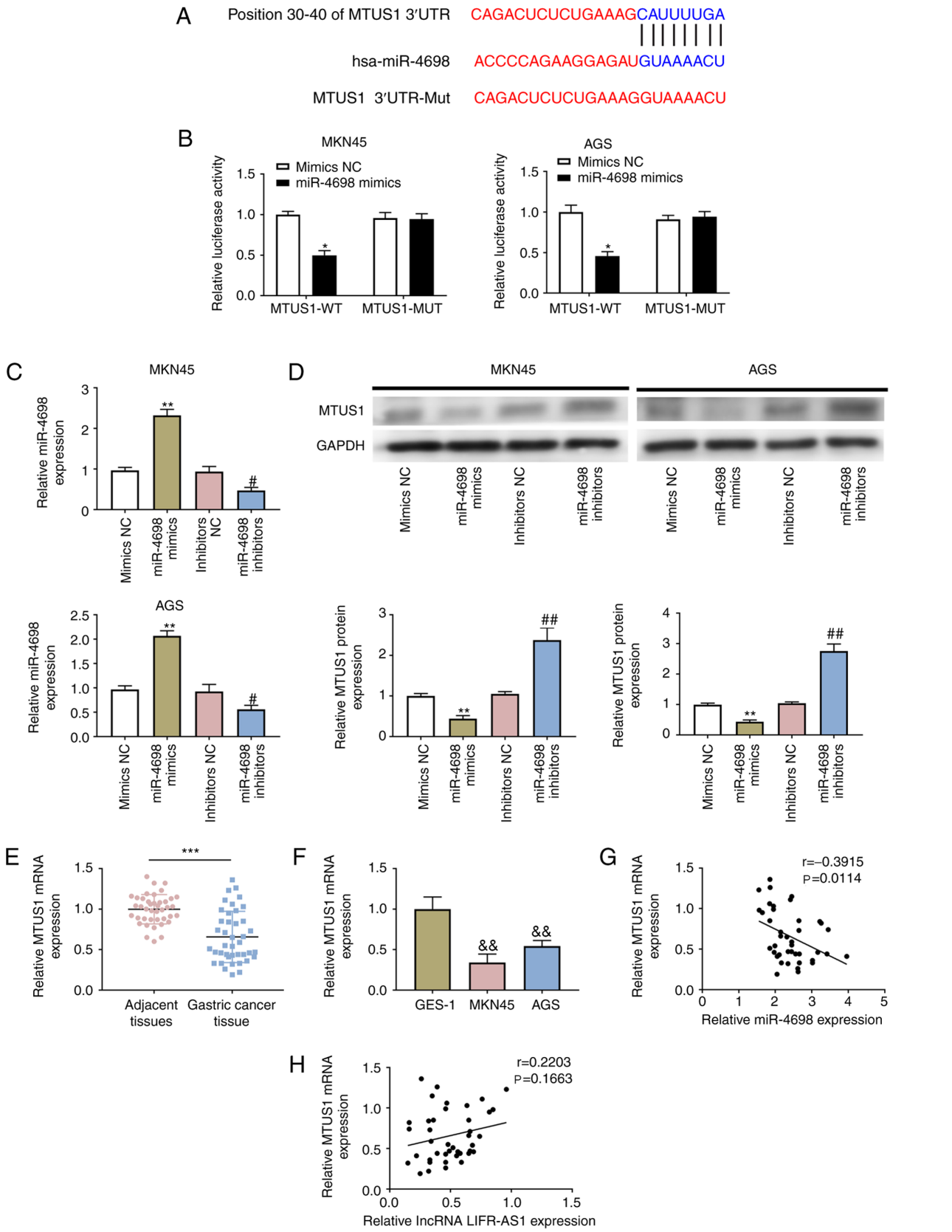

Figure 4. MTUS1 is a direct target of miR-4698 and is positively regulated by lncRNA LIFR-AS1. (A) The binding sequence between miR-4698 and MTUS1 was predicted using TargetScan algorithms. (B) Association between MTUS1 and miR-4698 was evaluated using a luciferase reporter system. The vectors of miR-4698 mimics and miR-4698 inhibitors as well as the corresponding controls (mimics NC and inhibitors NC) were transfected into GC (MKN45 and AGS) cell lines. (C) The expression of miR-4698 in above transfected cells was explored using RT-qPCR; (D) The expression of MTUS1 in above transfected cells was evaluated using western blotting. Expression of MTUS1 in (E) 41 pairs of GC tissues and matched adjacent tissues and (F) in GES-1 and GC (MKN45 and AGS) cell lines was examined using RT-qPCR. Interactions between MTUS1 and (G) miR-4698 or (H) lncRNA LIFR-AS1 were investigated using Pearson correlation analysis. *P<0.05 vs. MTUS1-WT+Mimics NC group, **P<0.01 vs. Mimics NC group, $^{\&\&}$P<0.01 vs. GES-1 group, ***P<0.001 vs. adjacent tissues group, $^{\#}$P<0.05 vs. inhibitors NC group, $^{\#\#}$P<0.01 vs. inhibitors NC group. MTUS1, microtubule-associated tumor suppressor 1; miR, microRNA; lnc, long non-coding; NC, negative control; GC, gastric carcinoma; GES-1, gastric mucosal epithelial cell line; RT-qPCR, reverse transcription-quantitative PCR.

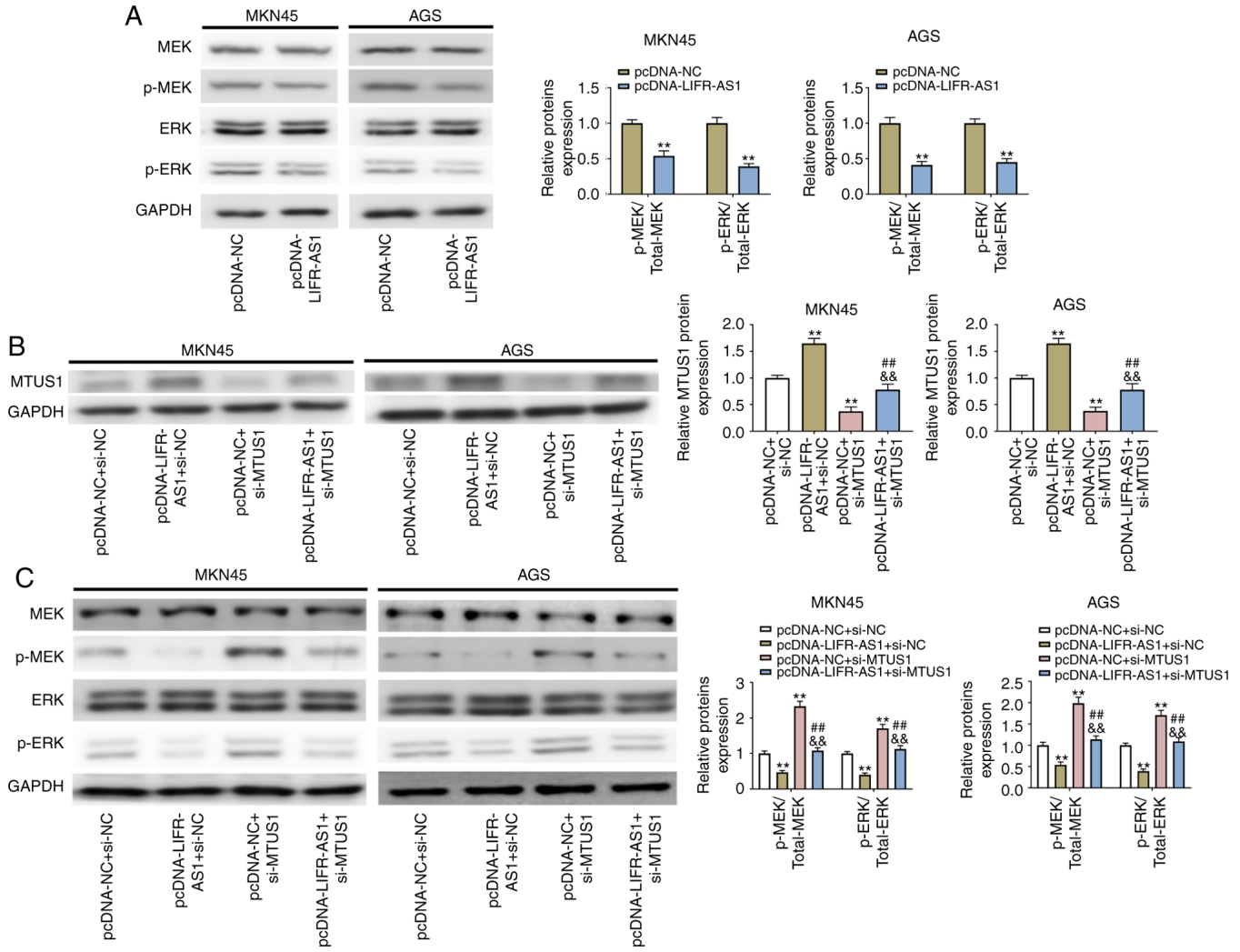

Figure 5. Overexpression of lncRNA LIFR-AS1 blocks the MEK/ERK pathway by regulating MTUS1. (A) The vectors of pcDNA-LIFR-AS1 and pcDNA-NC were transfected into GC (MKN45 and AGS) cell lines and the protein levels of p-MEK/MEK and p-ERK/ERK were examined using western blotting. (B) The expressed vectors of pcDNA-LIFR-AS1 and si-MTUS1 as well as the corresponding controls (pcDNA-NC and si-NC) were transfected into GC (MKN45 and AGS) cell lines and the expression of MTUS1 in the transfected cells examined using western blotting. (C) The vectors of pcDNA-LIFR-AS1, si-MTUS1 and the corresponding controls (pcDNA-NC and si-NC) were transfected into GC (MKN45 and AGS) cell lines and protein levels of p-MEK/MEK and p-ERK/ERK were examined using western blotting. **P<0.01 vs. pcDNA-NC group, $^{\#\#}$P<0.01 vs. pcDNA-LIFR-AS1+si-NC group, $^{\&\&}$P<0.01 vs. pcDNA-NC+si-MTUS1 group. lnc., long non-coding; MTUS1, microtubule-associated tumor suppressor 1; si, small interfering; NC, negative control; GC, gastric carcinoma; p-, phosphorylated.

LIFR-AS1 (Fig. 4H). Together, these results demonstrated that MTUS1 was a new predicated target gene of miR-4698 and was also positively regulated by lncRNA LIFR-AS1.

MEK/ERK pathway is counteracted by overexpression of lncRNA LIFR-AS1 via regulation of MTUS1. The signaling pathway regulation was assessed when cells were transfected with the expression vectors of pcDNA-LIFR-AS1 and si-MTUS1. It was noted that overexpressed lncRNA LIFR-AS1 inhibited p-MEK and p-ERK expression in GC (MKN45 and AGS) cell lines (P<0.05; Fig. 5A). si-MTUS1 vector was transfected into GC cells to inhibit MTUS1 expression and the relationship between lncRNA LIFR-AS1 and MTUS1 explored. It was observed that overexpressed lncRNA LIFR-AS1 upregulated MTUS1 expression in MKN45 and AGS cells (P<0.01; Fig. 5B). By contrast, silencing of MTUS1 increased p-MEK and p-ERK expression (P<0.05, Fig. 5C). Following pcDNA-LIFR-AS1 and si-MTUS1 co-transfection, the suppressed p-MEK and p-ERK expression in GC (MKN45 and AGS) cell lines was reversed by si-MTUS1 (P<0.01; Fig. 5C). On the basis of these results, it was hypothesized that the MEK/ERK pathway was hindered by overexpressed lncRNA LIFR-AS1 modulating MTUS1.

Overexpression of lncRNA LIFR-AS1 inhibits Cdc25B and inactivates Cdk1 signaling. The present study identified that overexpressed lncRNA LIFR-AS1 inhibited Cdc25B expression in GC (MKN45 and AGS) cell lines. The tyrosine phosphorylation of Cdk1 was induced by overexpression of lncRNA LIFR-AS1 in GC (MKN45 and AGS) cell lines (P<0.05; Fig. 6A). Following pcDNA-LIFR-AS1 and si-MTUS1 co-transfection, the suppressed Cdc25B expression and the induced tyrosine phosphorylation of Cdk1 in GC (MKN45 and AGS) cell lines was reversed by si-MTUS1 (P<0.01; Fig. 6B). These results demonstrated that the inhibition of Cdc25B activity by lncRNA LIFR-AS1 could contribute to Cdk1 tyrosine phosphorylation.

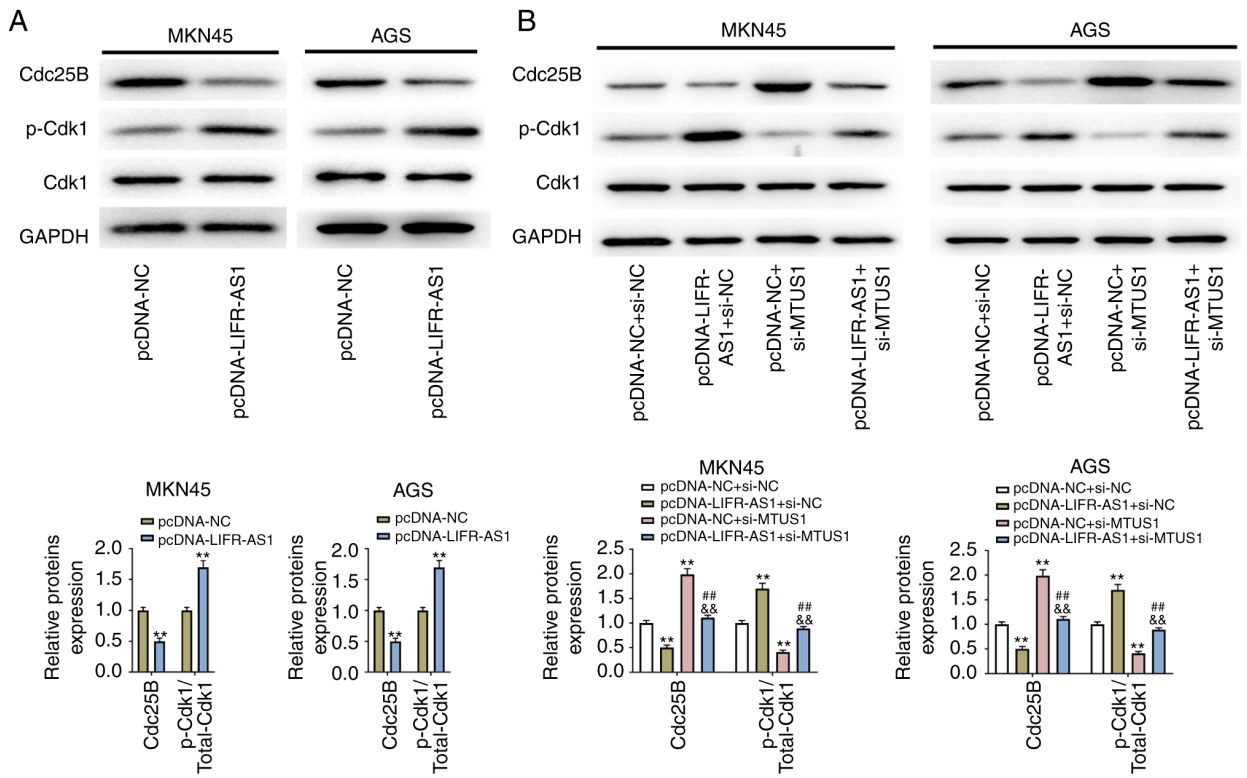

Figure 6. Overexpression of lncRNA LIFR-AS1 inhibits Cdc25B and inactivates Cdk1 signaling. (A) The vectors of pcDNA-LIFR-AS1 and pcDNA-NC were transfected into GC (MKN45 and AGS) cell lines, protein levels of Cdc25B and p-Cdk1 were examined using western blotting. (B) The vectors of pcDNA-LIFR-AS1, si-MTUS1 and the corresponding controls (pcDNA-NC and si-NC) were transfected into GC (MKN45 and AGS) cell lines, protein levels of Cdc25B and p-Cdk1 were examined using western blotting. **P<0.01 vs. pcDNA-NC group, $^{\#\#}$P<0.01 vs. pcDNA-LIFR-AS1+si-NC group, $^{\&\&}$P<0.01 vs. pcDNA-NC+si-MTUS1 group. lnc, long non-coding; Cdc, cell division cycle; Cdk, cyclin-dependent kinase; NC, negative control; GC, gastric carcinoma; si, small interfering; MTUS1, microtubule-associated tumor suppressor 1.

Discussion

GC has become one of the leading causes of mortality in individuals and its pathogenesis is still unclear (27). In the treatment of GC priority is given to surgical operation, whereas the unfavorable prognosis remains an enormous challenge in clinical practice. Due to their clinical potential in cancer treatment, lncRNAs have been extensively studied in GC (28). Wu *et al* (29) reported that lncRNA ZEB2-AS1 is upregulated in gastric cancer and affects cell proliferation and invasion via miR-143-5p/HIF-1α axis. Xiao *et al* (30) found that lncRNA MALAT1 increases the stemness of gastric cancer cells via enhancing SOX2 mRNA stability. However, the influence of lncRNA LIFR-AS1 in GC remains to be elucidated. The present study first investigated the functions of lncRNA LIFR-AS1 in GC cell proliferation and movement and uncovered the potential molecular mechanisms. Decreased expression of lncRNA LIFR-AS1 was identified in GC tissues and cell lines. Further study revealed that lncRNA LIFR-AS1 inhibited GC cell proliferation, migration and invasion through sponging miR-4698. In addition, MTUS1 was verified to be a target gene of miR-4698 and positively regulated by lncRNA LIFR-AS1. Furthermore, lncRNA LIFR-AS1 blocked the MEK/ERK pathway by mediating MTUS1.

lncRNA LIFR-AS1 is derived from the antisense transcription of the *LIFR* gene located on human chromosome 5p13.1, which serves a crucial role in the pathogenesis of a number of illnesses (31). A previous study revealed that lncRNA LIFR-AS1 negatively correlates with tumor size in uterine fibroids (32). Additionally, Wang *et al* (13) found that lncRNA LIFR-AS1 is downregulated in breast cancer. In accordance with that study, the present study also observed decreased lncRNA LIFR-AS1 expression in GC tissues and cell lines. Xu *et al* (14) explored the effects of lncRNA LIFR-AS1 on breast cancer cell proliferation and migration and verify its inhibitory role in breast cancer progression. On the basis of that research, the functions of lncRNA LIFR-AS1 in GC cell proliferation, migration and invasion were further investigated. The present study also demonstrated the suppressed functions of lncRNA LIFR-AS1 in GC cell proliferation, migration and invasion. These findings suggested that lncRNA LIFR-AS1 participated in the control of the GC progression.

Previous studies have demonstrated the vital regulatory functions of lncRNA-miRNA network in types of human cancers, such as diabetic pancreatic cancer and colorectal cancer (33,34). In the field of GC, lncRNA gastric cancer-related lncRNA1 has been reported to modulate GC cell proliferation and metastasis by sponging miR-885-3p (35). lncRNA XIST promotes GC cell growth and invasion by regulating miR-497 (36). miR-4698 is a novel miRNA and its downregulation is reported in chronic obstructive pulmonary disease (37). Similarly, a profile of miRNAs by microarrays in Liu *et al* (38) demonstrates that miR-4698 expression is upregulated in gastric cancer stem cells. Considering its abnormal expression in different diseases, the present study attempted to uncover the influence of miR-4698 in lncRNA LIFR-AS1-affected GC cell proliferation, migration and

invasion. Upregulated expression of miR-4698 was observed in GC tissues and cell lines. In addition, a reciprocal suppression between miR-4698 and lncRNA LIFR-AS1 was identified. Thus, it was concluded that lncRNA LIFR-AS1 exerted inhibitory functions in GC cell proliferation, migration and invasion through sponging miR-4698.

MTUS1 is reported to locate on the reverse strand of the chromosome 8p22, which can encode angiotensin II AT2 receptor-interacting proteins (39). As a tumor suppressor, MTUS1 has been investigated in colon and prostate cancer and its aberrant expression is associated with the development of these cancers (40,41). The present study also observed the downregulated expression of MTUS1 in GC tissues and cell lines. In a previous study, silencing of MTUS1 facilitates GC cell growth and metastasis (42). The results of the present study confirmed that MTUS1 was a target gene of miR-4698. In addition, a positive correlation between MTUS1 and lncRNA LIFR-AS1 was identified. Given the relationship of lncRNA LIFR-AS1, miR-4698 and MTUS1, it was hypothesized that MTUS1 probably joined in modulating the functions of lncRNA LIFR-AS1 in GC.

MEK/ERK is a highly conserved cell signaling pathway, existing in eukaryotic cells, which regulates diverse cell behaviors in human cancers (43,44). The MEK/ERK pathway participates in adjusting the cisplatin resistance in GC cells (45) and inactivation of the MEK/ERK pathway can affect the proliferative ability of GC cells (46). Several lncRNAs affect cancer cell proliferation and metastasis by controlling the MEK/ERK pathway (47,48). However, whether lncRNA LIFR-AS1 regulates the MEK/ERK pathway to restrain GC cell proliferation and movement remains unreported. The present study showed that overexpressed lncRNA LIFR-AS1 inhibited the MEK/ERK pathway. The process was reversed by silencing of MTUS1. These findings corroborated that the MEK/ERK pathway was inhibited by overexpression of lncRNA LIFR-AS1 via modulating MTUS1.

Cdc25 serves an important role in transitions between cell-cycle phases by dephosphorylating and activating Cdks (49). ERK-MAP kinases are directly involved in activating Cdc25 during the G(2)/M transition (50). A previous study reported that small-molecule Cdc25 inhibitors inhibit Cdc25 enzymatic activities and induce tyrosine phosphorylation of the Cdc25-targeted Cdks (51). The present study identified that lncRNA LIFR-AS1 inhibited Cdc25B activity and contributed to Cdk1 tyrosine phosphorylation and cell growth inhibition.

The present study had some limitations. First, animal experiments to investigate the roles of lncRNA LIFR-AS1 in GC were not performed. Second, the role of lncRNA LIFR-AS1 on tumor cell apoptosis was unknown. Third, the underlying molecular mechanism of lncRNA LIFR-AS1 downregulation in GC cells was not elucidated.

In summary, the present study showed that lncRNA LIFR-AS1 was downregulated in GC tissues and was associated with the prognosis of patients with GC. lncRNA LIFR-AS1 inhibited GC cell proliferation, migration and invasion. Results revealed that lncRNA LIFR-AS1 interacted with miR-4698 to regulate MTUS1 expression. These results provided a new lncRNA-directed therapeutics for GC. However, the detailed effects of miR-4698 and MTUS1 on GC cell behaviors remain to be elucidated.

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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

JC and JZ conceived and designed the study. JZ, XL, LF, NZ and JY performed the study, collected the data and analyzed the data. JC wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All patients personally signed an informed consent. The protocol of the present study was approved by the Ethics Committee of Cangzhou People's Hospital (approval number AF/SC-08/02.0).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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