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Long non-coding RNA AFAP1-AS1 promotes proliferation and migration of gastric cancer by downregulating KLF2

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Abstract. – OBJECTIVE: To clarify the function of actin filament associated protein 1-antisense RNA1 (AFAP1-AS1) to promote the proliferation and migration of gastric cancer (GC) cells by downregulating Krüppel-like factor 2 (KLF2).

MATERIALS AND METHODS: Expression level of AFAP1-AS1 in GC tissues and matched paracancerous tissues was determined by quantitative real-time polymerase chain reaction (qRT-PCR). Besides, its level in GC either with lymphatic metastasis or not, and those in different tumor stages were determined as well. Regulatory roles of AFAP1-AS1 in cellular behaviors of GC cells were evaluated by functional experiments. The ability of AFAP1-AS1 to recruit EZH2 was evaluated through chromatin immunoprecipitation (ChIP) assay. The expression level of KLF2 in GC cells influenced by AFAP1-AS1 and EZH2 was detected by Western blot. Finally, a series of rescue experiments were conducted to clarify the role of AFAP-AS1/KLF2 in GC cell performances.

RESULTS: AFAP1-AS1 was upregulated in GC tissues, and its expression in lymph node metastasis and progressive gastric cancer tissues were much higher. Knockdown of AFAP1-AS1 reduced the viability, proliferative and migratory abilities, but induced apoptosis of GC cells. AFAP1-AS1 was verified to bind to EZH2. After knockdown of AFAP1-AS1, the ability of AFAP1-AS1 to recruit EZH2 was remarkably attenuated. Knockdown of AFAP1-AS1 or EZH2 upregulated KLF2 expression in GC cells. Notably, knockdown of KLF2 partially reversed the effect of AFAP1-AS1 on GC cell performances.

CONCLUSIONS: LncRNA AFAP1-AS1 accelerates the proliferative and migratory abilities of GC cells by downregulating the expression of KLF2, thus promoting the progression of GC.

Key Words:
Gastric cancer, AFAP1-AS1, KLF2.

Introduction

Gastric cancer (GC) is the fifth most prevalent cancer worldwide and the third leading cause of cancer-related deaths, posing a great public health problem[1]. Annually, there are approximately one million newly onsets of GC and about 700,000 people die of GC, accounting for one-tenth of all cancer-related deaths[2]. The lack of effective screening methods, and the apparent absence of early-stage symptoms result in the high mortality rate of GC. Most GC patients are already in the advanced stage when they are first diagnosed. The therapeutic effect of conventional chemotherapy and surgical treatment for advanced GC is limited, leading to a poor prognosis[3]. Therefore, prevention and diagnosis as early as possible have important guiding significance for the clinical treatment of GC.

Actin filament associated protein 1-antisense RNA1 (AFAP1-AS1) is a long non-coding RNA of 6810 nt, exerting a vital role in the development of esophageal cancer, nasopharyngeal cancer, lung cancer, pancreatic cancer, and other tumors[4-9]. Current studies[10] have shown the promotive effect of AFAP1-AS1 on the tumorigenesis of GC, but the related mechanism needs to be further studied.

Krüppel-like factors (KLFs) are transcription factors of zinc finger-like structure[11], which is a subfamily of zinc finger protein superfamily. At present, 17 KLF factors have been found

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in mammals. As a member of the KLFs family, KLF2 has been initially found in human lung tissues[12]. KLF2 is a protein of 354 amino acids, located on chromosome 19p13.1, and has more than 85% homology with mouse genes. Recently, it has been reported that lncRNA DLEU1 can suppress KLF2 expression through epigenetic repression, thereby promoting GC cells to proliferate[13]. However, whether the expression level of KLF2 could be affected by AFAP1-AS1 has not been reported. Therefore, this study aims to uncover the potential effects of AFAP1-AS1 and KLF2 on the occurrence and development of GC. Our research provides novel clinical ideas for finding diagnostic and therapeutic targets for GC.

Materials and Methods

Cell Culture and Transfection

Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; HyClone, South Logan, UT, USA), 1% penicillin-streptomycin in the constant temperature incubator at 37°C and 5% CO_2. Fourth or fifth-generation cells in good growth state were selected for cell transfection. They were pre-seeded in a 6-well plate and incubated to 80% of confluence. Cells were transfected with sh-NC, sh-AFAP1-AS1 1#, sh-AFAP1-AS1 2#, sh-AFAP1-AS1 3#, sh-EZH2 or sh-AFAP1-AS1 1#+sh-KLF2 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Sequences of transfection plasmids were as follows: sh1AFAP1-AS11# forward, 5′-CCGGAGCGGTCTCAGCCGAATGACTCTCGAGAGTCATTCGGCTGAGACCGCTTTTTTG-3′ and reverse, 5′-AATTCAAAAAAGCGGTCTCAGCCGAATGACTCTCGAGAGTCATTCGGCTGAGACCGCT-3′; sh-AFAP1-AS12# forward, 5′-CCGGAACACCAATCCCAAGAGGTGACTCGAGTCACCTCTTGGGATTGGTGTTTTTTTG-3′ and reverse, 5′-AATTCAAAAAAACACCAATCCCAAGAGGTGACTCGAGTCACCTCTTGGGATTGGTGTT-3′; sh-NC, forward, 5′-CCGGTTTCTCCGAACGTGTCACGTCTCGAGACGTGACACGTTCGGAGAATTTTTG-3′ and reverse, 5′-AATTCAAAAAGTTCTCCGAACGTGTCACGTCTCGAGACGTGACACGTTCGGAGAA-3′; sh-EZH2 forward, 5′-AGAGGTACCGGACGAAGAATAATCATGG-3′ and reverse, 5′-TAGCTCGAGGGTAGCAGATGTAAGG-3′; sh-KLF2 forward, 5′-GATCCGCGCACCCACGACGACCTCAATTCAAGAGATTGAGGTCGTCGTCGGTGCCGTTTTTTC-3′ and reverse, 5′-AATTGAAAAAACGGCACCGACGACGACCTCAATCTCTTGAATTGAGGTCGTCGTCGGTGCCGCG-3′.

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Total RNA extraction was performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and the expression levels of target genes were determined using the PCR kit (TaKaRa, Otsu, Shiga, Japan). The primer sequences were as follows: AFAP1-AS1 forward, 5'-GGAGTGACGGCATCCAACTC-3', reverse, 5'-GTCATCCCTGTCCCTGGTTC-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward, 5'-CAATGACCCCTTCATTGACC-3', reverse, 5'-GACAAGCTTCCCGTTCTCAG-3'.

Cell Counting Kit-8 (CCK-8)

Cells were pre-seeded into a 96-well plate with 1×10^6 cells per well. After cell culture for 6, 24, 48, 72, and 96 h, 10 μl of CCK-8 (Dojindo Laboratories, Kumamoto, Japan) was added into each well and incubated at 37°C under 5% CO_2 for 1 h. The absorbance at 450 nm was measured with an enzyme-standard instrument.

Flow Cytometry

Cells in a good growth state were harvested and the concentration was adjusted to 1×10^6/mL. After 24 h of culture, cells were prepared for single-cell suspension. Cells were double-stained with 5 μL of Annexin V and 5 μL of fluorescein isothiocyanate (FITC) in the dark for 10 min. The apoptotic rate was detected using flow cytometry (Partec AG, Arlesheim, Switzerland).

5-Ethynyl-2′- Deoxyuridine (EdU) Assay

Transfected cells were pre-seeded in the 96-well plate with 5×10^3 cells per well. Cells were labeled with 50 μmol/L EdU at 37°C for 2 h. Subsequently, cells were subjected to 30-min fixation in 4% paraformaldehyde and 20-min permeation in phosphate-buffered saline (PBS) containing 0.5% Triton-100. After washing with PBS containing 3% bovine serum albumin (BSA), 100 μL of the dying solution was applied per well for 1 h incubation in the dark and cells were counter-stained with 100 μL 4',6-diamidino-2-phenylindole (DAPI) (5 μg/mL) for 30 min. The ratio of EdU-positive cells was calculated.

Transwell Assay

3×10^4 cells were inoculated in the upper side of the transwell chamber (Millipore, Billerica, MA,

USA). In the bottom side, 600 μL of medium containing 20% FBS was applied. After 48 h of incubation, invasive cells were fixed in methanol for 10-15 min, dyed with 0.5% crystal violet for 20 min and counted using a microscope. 5 random fields were selected for calculating the penetrating cells.

RNA Immunoprecipitation (RIP)

Cells were collected and treated according to the procedures of Millipore Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA). The cell lysate was incubated with anti-EZH2, anti-H3K4me3, or IgG antibody at 4°C for 6 h. A protein-RNA complex was captured and digested with 0.5 mg/ml proteinase K containing 0.1% sodium dodecyl sulphate (SDS) to extract RNA. The magnetic beads were repeatedly washed with RNA protein immunoprecipitation (RIP) washing buffer to remove non-specific adsorption as much as possible. Finally, the extracted RNA was subjected to mRNA level determination using qRT-PCR.

Chromatin Immunoprecipitation (ChIP)

Cells were subjected to 10-min cross-link with 1% formaldehyde at room temperature. Subsequently, cells were lysed using lysis buffer and sonicated for 30 min. Finally, the sonicated lysate was immuno-precipitated with antibodies and IgG. EZH2 forward, 5'-TGCACATCCTGACTTCTGTG-3' and reverse, 5'- AAGGGCATTCACCAACTCC-3'.

Western Blot

Proteins were extracted from cells and loaded for electrophoresis and transferred on a polyvinylidene difluoride (PVDF) membranes. After blocking in 5% skim milk for 2 h, membranes were subjected to incubation with primary antibodies at 4°C overnight and secondary antibodies for 2 h. Bands were exposed by enhanced chemiluminescence (ECL) and analyzed by Image Software (NIH, Bethesda, MD, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA) was used for data analyses. Data were expressed as mean ± standard deviation. The intergroup differences were analyzed by the *t*-test. $p<0.05$ was considered statistically significant.

Results

AFAP1-AS1 Was Upregulated in GC Tissues

AFAP1-AS1 was highly expressed in GC tissues relative to matched normal ones (Figure 1A). AFAP1-AS1 expression was higher in GC tissues with lymphatic metastasis and progressive GC (Figure 1B and 1C). The above results indicated that AFAP1-AS1 was upregulated in GC tissues.

Knockdown of AFAP1-AS1 Promoted Apoptosis of GC Cells

QRT-PCR results revealed that AFAP1-AS1 was highly expressed in GC cell lines as well, with the highest expression in SGC-7901 and AGS cells (Figure 2A). Transfection of sh-AFAP1-AS1 1# or sh-AFAP1-AS1 2# markedly downregulated AFAP1-AS1 level in GC cells (Figure 2B).

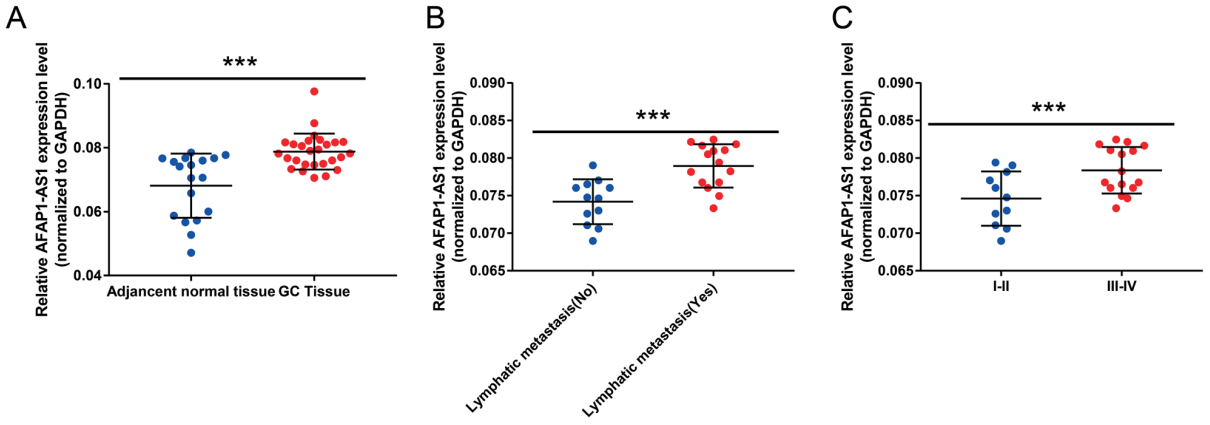

Figure 1. AFAP1-AS1 was upregulated in GC tissues. **A,** Relative level of AFAP1-AS1 in GC tissues and adjacent normal tissues. **B,** Relative level of AFAP1-AS1 in GC with lymphatic metastasis or not. **C,** Relative level of AFAP1-AS1 in GC with stage I-II or stage III+IV.

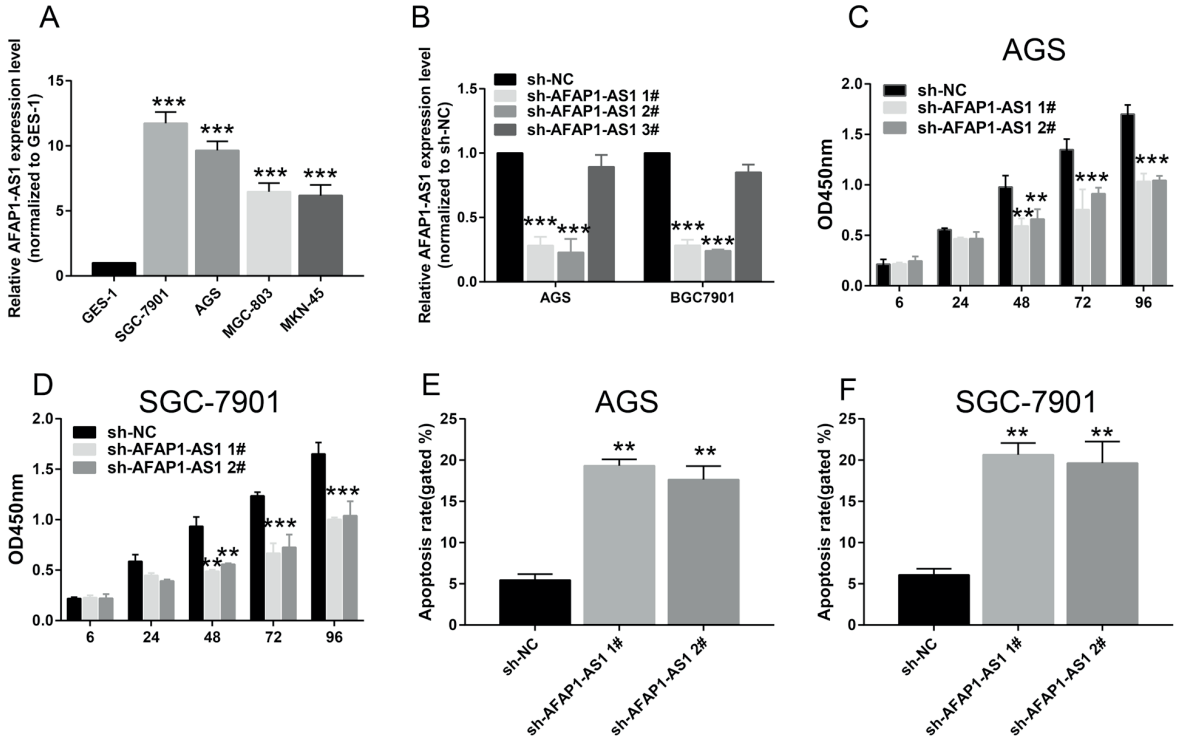

Figure 2. Knockdown of AFAP1-AS1 induced apoptosis of GC cells. **A,** Relative level of AFAP1-AS1 in GES-1 cell line and GC cell lines. **B,** Transfection efficacy of sh-AFAP1-AS1 1#, sh-AFAP1-AS1 2# and sh-AFAP1-AS1 3# in AGS and SGC-7901 cells. **C,** CCK-8 assay showed the viability in AGS cells transfected with sh-NC, sh-AFAP1-AS1 1# or sh-AFAP1-AS1 2#. **D,** CCK-8 assay showed the viability in SGC-7901 cells transfected with sh-NC, sh-AFAP1-AS1 1# or sh-AFAP1-AS1 2#. **E,** Apoptotic rate in AGS cells transfected with sh-NC, sh-AFAP1-AS1 1# or sh-AFAP1-AS1 2#. **F,** Apoptotic rate in SGC-7901 cells transfected with sh-NC, sh-AFAP1-AS1 1# or sh-AFAP1-AS1 2#.

CCK-8 experiments showed that cell viability was remarkably inhibited at 48, 72, and 96 h after the knockdown of AFAP1-AS1 (Figure 2C, 2D). Notably, the apoptotic level was elevated by the transfection of sh-AFAP1-AS1 1# or sh-AFAP1-AS1 2# in GC cells (Figure 2E, 2F). These results illustrated that AFAP1-AS1 elevated cell viability and inhibited apoptosis in GC.

Knockdown of AFAP1-AS1 Attenuated GC Cells to Proliferate and Invade

EdU assay revealed the decreased number of EdU-positive cells after the knockdown of AFAP1-AS1 in GC cells, suggesting the inhibited proliferative ability (Figure 3A, 3B). Meanwhile, the migratory ability of GC cells was reduced by transfection of sh-AFAP1-AS1 1# or sh-AFAP1-AS1 2# as the transwell assay indicated (Figure 3C, 3D).

AFAP1-AS1 Mediated Epigenetic Repression of KLF2 Via Recruitment of EZH2

Through online bioinformatics analysis (http://pridb.gdcb.iastate.edu/RPSeq/references.php), we predicted the binding between AFAP1-AS1 and KLF2 (Figure 4A). RIP further validated the binding of them (Figure 4B). After AFAP1-AS1 knockdown, the ability of AFAP1-AS1 to recruit EZH2 was significantly downregulated (Figure 4C and 4D). Knockdown of AFAP1-AS1 or EZH2 both upregulated protein expression of KLF2, indicating that KLF2 expression was regulated by AFAP1-AS1 recruitment of EZH2 (Figure 4E and 4F).

Knockdown of KLF2 Partially Reversed the Influence of AFAP1-AS1 on GC Cell Performances

To further investigate the regulatory interaction between AFAP1-AS1 and KLF2, KLF2 expression was significantly downregulated after transfection of sh-KLF2 (Figure 5A), indicating the transfection was efficient. Knockdown of KLF2 partially reversed the decline in cell viability and migratory ability caused by the knockdown of AFAP1-AS1 (Figure 5B-5E). These results suggested that AFAP1-AS1 affected cell viability and migratory ability by down-regulating KLF2 expression in GC cells.

Discussion

The incidence of GC in China ranks second in the world only secondary to Japan, with almost 400,000 newly cases detected annually, accounting for 42% of the global GC cases. It ranks second in the tumor incidence in our country, and the third in tumor death, leading to about 300,000 death cases[14]. It is estimated that the 5-year survival of postoperative progressive GC is less than 30%[15]. Therefore, it is particularly important to clarify the pathogenesis of GC to find the screening basis and therapeutic targets for improving the prognosis of GC patients.

LncRNA was initially regarded as a by-product of RNA polymerase II transcription without any biological function. Several studies[16] nowadays have identified the vital role of lncRNAs in tumorigenesis. Many lncRNAs have been proved to be related to GC. For example, lncRNA

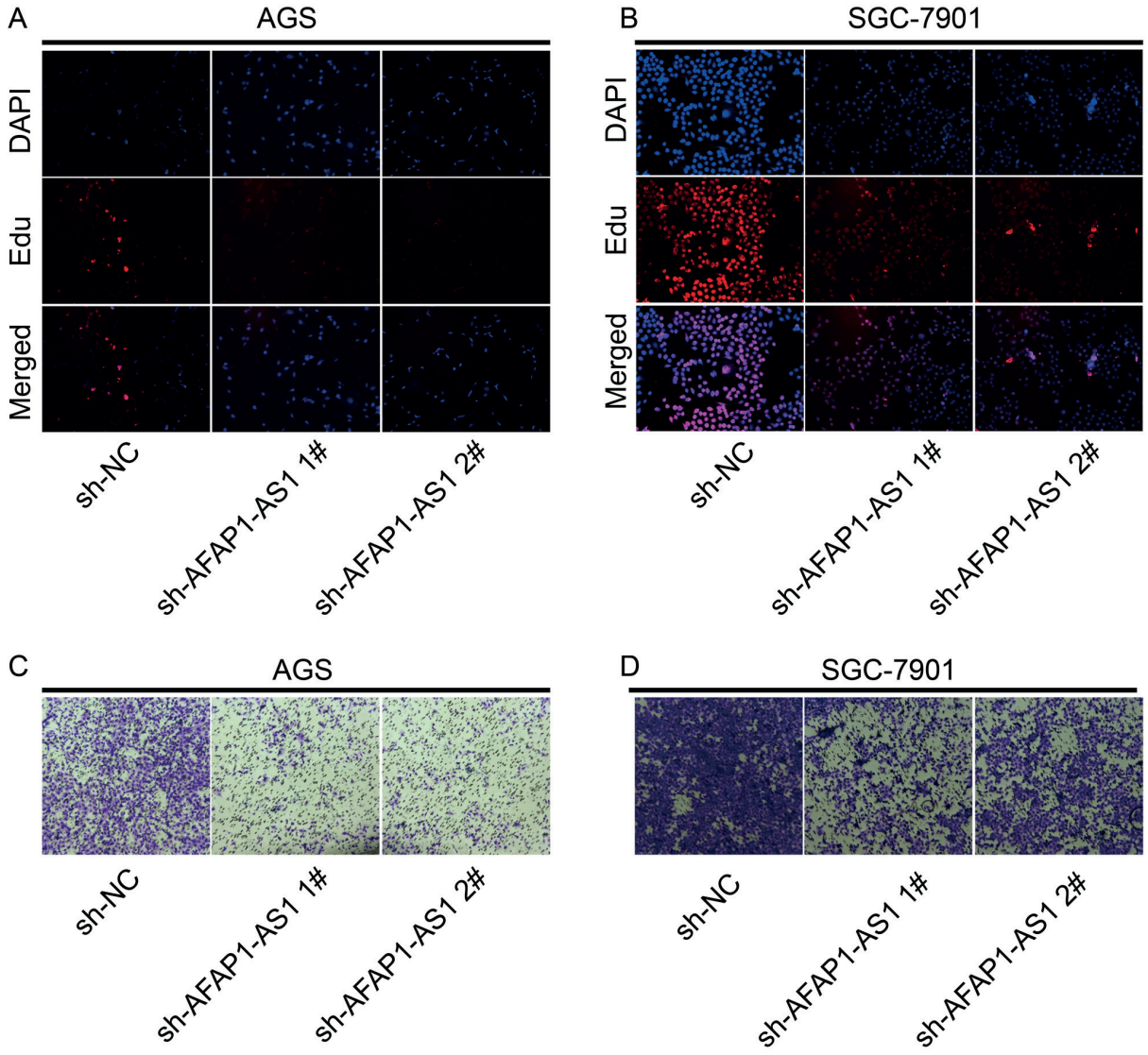

Figure 3. Knockdown of AFAP1-AS1 suppressed proliferation and invasion of GC cells. **A,** EdU assay showed EdU-positive cells in AGS cells transfected with sh-NC, sh-AFAP1-AS1 1# or sh-AFAP1-AS1 2# (DAPI was stained blue and EdU-positive cells were stained red) (magnification: 200×). **B,** EdU assay showed EdU-positive cells in SGC-7901 cells transfected with sh-NC, sh-AFAP1-AS1 1# or sh-AFAP1-AS1 2# (DAPI was stained blue and EdU-positive cells were stained red) (magnification: 200×). **C,** Transwell assay showed invasive cells in AGS cells transfected with sh-NC, sh-AFAP1-AS1 1# or sh-AFAP1-AS1 2# (magnification: 40×). **D,** Transwell assay showed invasive cells in SGC-7901 cells transfected with sh-NC, sh-AFAP1-AS1 1# or sh-AFAP1-AS1 2# (magnification: 40×).

LOC554202 promotes proliferation and migration of gastric cancer cells by regulating p21 and E-cadherin[17]. LncRNA SNHG6 induces the epithelial-mesenchymal transformation (EMT) of GC cells by silencing the p27 signaling pathway, thus aggravating the disease progression[18]. LncRNA SNHG1 accelerates GC cells to proliferative by regulating the expression of DNMT1[19]. Ye et al[20] have shown that AFAP1-AS1 is upregulated in pancreatic ductal adenocarcinoma (PDAC), which stimulates the proliferative, invasive, and infiltration capacities of PDAC. The expression of AFAPl-ASl is upregulated in nasopharyngeal carcinoma (NPC) as well, which stimulates the invasive ability of NPC by regulating the integrity of actin filaments[21]. AFAP1-AS1 can promote the malignant phenotypes of GC cells[22], and is related to the survival rate of patients. This investigation found that the expression of lncRNA AFAP1-AS1 was overexpressed in GC, suggesting that AFAP1-AS1 may be involved in the occurrence of GC. In addition, we found that the knockdown of AFAP1-AS1 in GC cells suppressed proliferative and invasive abilities, but induced apoptosis.

KLF2 remains a low level in a variety of malignant tumors as a tumor suppressor[23]. The expression level of KLF2 in non-small cell lung cancer (NSCLC) is markedly lower relative to adjacent normal tissues, and NSCLC patients with a low level of KLF2 present poor prognosis[24]. It is reported[25] that KLF2 shows a low abundance in many types of tumor cells. Li et al[13] demonstrated that lncRNA DLEU1 accelerates GC cells to proliferate by downregulating KLF2. Hence, KLF2 is believed to exert a tumor-suppressor effect. Notably, our work found that KLF2 could partially reverse the regulatory role of AFAP1-AS1 in GC cell performances.

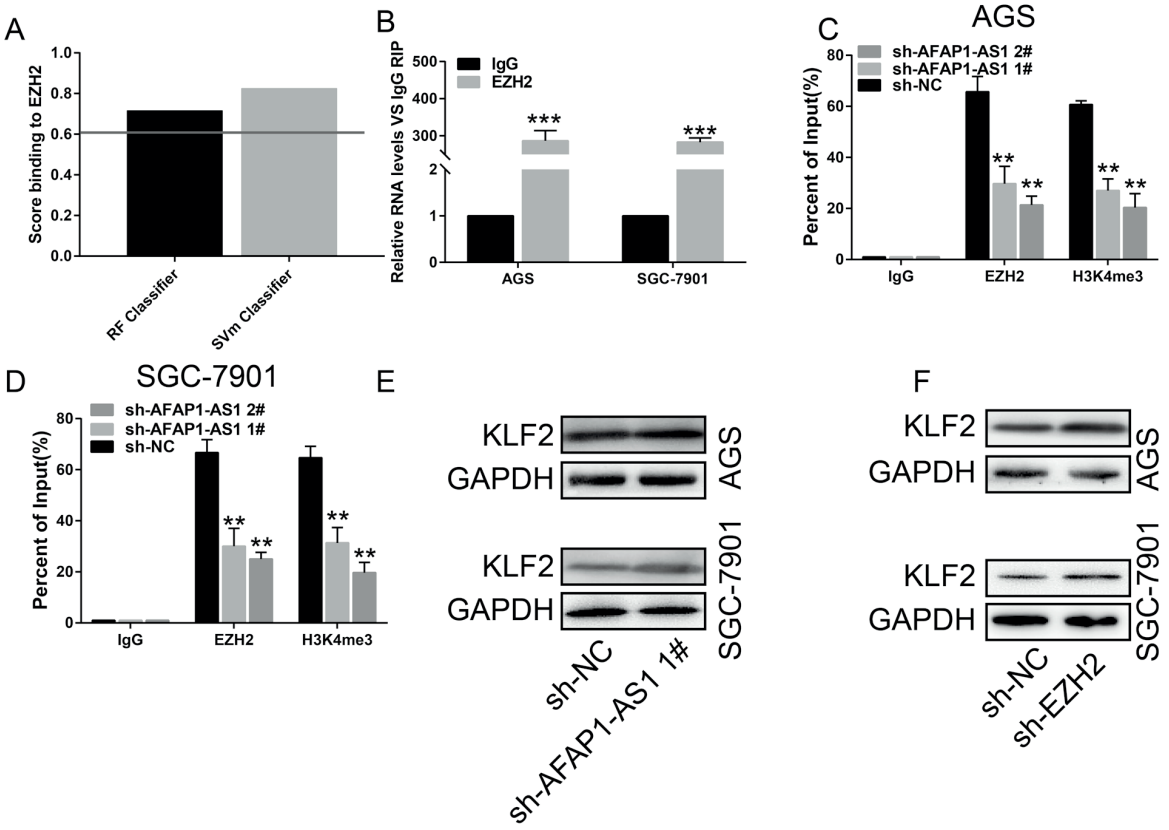

Figure 4. AFAP1-AS1 mediated epigenetic repression of KLF2 *via* recruitment of EZH2. **A,** RF and SVM classifier showed score binding to EZH2. **B,** Immuno-precipitates of IgG and EZH2 in AGS and SGC-7901 cells. **C,** Percentage of input in IgG, EZH2 and H3K4me3 after transfection of sh-NC, sh-AFAP1-AS1 1# or sh-AFAP1-AS1 2# in AGS cells. **D,** Percentage of input in IgG, EZH2 and H3K4me3 after transfection of sh-NC, sh-AFAP1-AS1 1# or sh-AFAP1-AS1 2# in SGC-7901 cells. **E,** Western blot analysis of KLF2 in AGS and SGC-7901 cells transfected with sh-NC or sh-AFAP1-AS1 1#. **F,** Western blot analysis of KLF2 in AGS and SGC-7901 cells transfected with sh-NC or sh-EZH2.

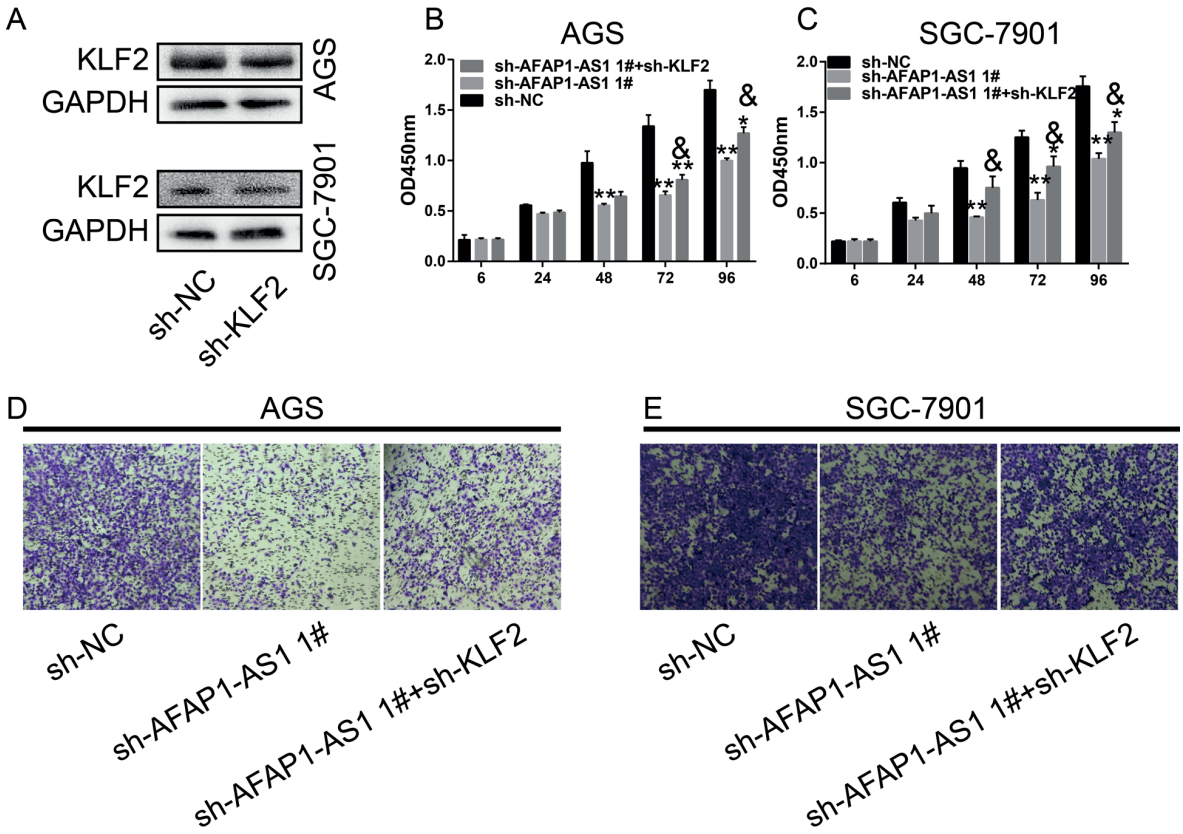

Figure 5. Knockdown of KLF2 partially reversed the effect of AFAP1-AS1 on GC cell performances. **A,** Transfection efficacy of sh-KLF2 in AGS and SGC-7901 cells. **B,** CCK-8 assay showed the viability in AGS cells transfected with sh-AFAP1-AS1 1#+sh-KLF2, sh-AFAP1-AS1 1# or sh-NC. **C,** CCK-8 assay showed the viability in SGC-7901 cells transfected with sh-AFAP1-AS1 1#+sh-KLF2, sh-AFAP1-AS1 1# or sh-NC. **D,** Transwell assay showed the invasion in AGS cells transfected with sh-AFAP1-AS1 1#+sh-KLF2, sh-AFAP1-AS1 1# or sh-NC (magnification: 40×). **E,** Transwell assay showed the invasion in SGC-7901 cells transfected with sh-AFAP1-AS1 1#+sh-KLF2, sh-AFAP1-AS1 1# or sh-NC (magnification: 40×).

Conclusions

In sum, AFAP1-AS1 promoted GC cells to proliferate and invade via downregulating KLF2. Our research provides a novel therapeutic target for GC treatment.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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