Long noncoding AFAP1-antisense RNA 1 is upregulated and promotes tumorigenesis in gastric cancer

FEI YE, YI GONG, XIANGHENG CHEN, MEIYING YU, ZHONGKUN ZUO, DONGNI PEI, WEI LIU, QUNWEI WANG, JUN ZHOU, LUNXI DUAN, LEIYI ZHANG, XIAOJING LI, TENGLONG TANG and JIANGSHENG HUANG

Department of Minimally Invasive Surgery, The Second Xiangya Hospital of Central South University, Changsha, Hunan 410011, P.R. China

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Abstract. Long noncoding RNA serves important roles in gastric cancer (GC). However, the prognostic significance and tumorigenesis effect of AFAP1-antisense RNA 1 (AS1) in GC remain to be clarified. The present study was conducted in order to determine the expression level of AFAP1-AS1 by reverse transcription-quantitative polymerase chain reaction. It was demonstrated that AFAP1-AS1 expression level was higher in GC tissues in comparison with adjacent tissues. By analyzing 66 GC tissue specimens, AFAP1-AS1 expression level was found to be markedly associated with tumor size, clinical stage and differentiation. By performing multivariate Cox regression test, AFAP1-AS1 expression level was confirmed to be an independent factor for poor prognosis in patients with GC. Furthermore, SGC-7901 and BGC-823 cells were used for further investigation following transfection of an AFAP1-AS1 short hairpin RNA lentiviral vector. Knockdown of AFAP1-AS1 significantly inhibited GC cell proliferation, migration and invasion abilities in vitro. Finally, nude mice experiments confirmed that downregulation of AFAP1-AS1 in GC cells suppressed tumor growth in vivo. In conclusion, the results of the present study suggested that AFAP1-AS1 may serve as a valuable prognostic indicator and therapeutic target for GC.

Introduction

Gastric cancer (GC) is a major type of digestive tract tumor (1). According to statistical studies ~951,000 novel cases are diagnosed, and ~723,000 mortalities occur each year worldwide (2,3). There has been a significant rise of GC cases and GC associated mortalities over the past decades, accounting for ~8.8% of the mortality rate worldwide (4). Surgical resection is one of the most important methods for early gastric cancer treatment (5). At present, due to diagnostic and therapeutic advancements, the survival rate of patients with GC has increased (6). However, poor prognosis is still observed in patients with GC (7). The overall five-year survival rate of patients with GC remains low (8). Further study investigating the molecular mechanisms underlying GC tumorigenesis may provide promising therapeutic targets for GC.

Recent studies have revealed a large-scale regulatory network generated by non-coding RNAs, including long noncoding RNAs (lncRNAs) and microRNAs (miRNAs) (9,10). lncRNAs are conserved non-coding RNAs of >200 nucleotides (11). An increasing amount of evidence has indicated that dysregulation of lncRNAs is involved in numerous types of cancer, including GC (12). A series of lncRNAs involved in GC tumor development has been revealed by previous studies (13). It has been demonstrated that the expression level of IncRNA AFAP1-antisense RNA 1 (AS1) was significantly upregulated (~45-fold) in 3 gastric cancer tissues compared with in normal tissues by lncRNA microarray analysis (14), which indicated that AFAP1-AS1 may function as a tumor promoter in GC. To the best of our knowledge, the associations between AFAP1-AS1 expression level and clinicopathological parameters in patients with GC, and the potential mechanisms underlying the role of AFAP1-AS1 in GC tumorigenesis, have not yet been studied. The present study aimed to detect the expression level of AFAP1-AS1 in GC tissues and cells and confirm whether AFAP1-AS1 serves an important role in GC development.

Materials and methods

Tissue samples and cell lines. The present study was approved by the Ethics Committee of The Second Xiangya Hospital of Central South University (Changsha, China), and written informed consent was obtained from all patients prior to enrollment in the present study. Criteria for accepting patients were as follows: i) Patients were treated with gastrectomy at
The Second Xiangya Hospital of Central South University; ii) pathological confirmation of GC following surgery; iii) no preoperative anticancer treatment was received by patients with GC; iv) patients exhibited no other cancer history; and v) the adjacent normal tissues were located ≥2 cm away from the tumor. A total of 10 pairs of human GC tissues and corresponding matched normal tissues, and 56 samples of unmatched GC tissues were selected according to the inclusion criteria. A total of 66 patients with GC who underwent gastrectomy from January 2014 to May 2016 were prospectively enrolled. The median follow-up period of patients with GC was 13 months.

GES-1 human gastric epithelial cells and HGC-27, MGC-803, BGC-823 gastric cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). SGC-7901 cell lines were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). MGC-803, BGC-823 and SGC-7901 cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and GES-1 and HGC-27 cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.). All media were supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin mixture (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C in a humidified environment containing 5% CO₂.

Transfection and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). pGreenPuro™ (human cytomegalo-virus) short hairpin (sh)RNA vectors were inserted into two shRNA-AFAP1-AS1 or scrambled control shRNA (shNC) vectors (sh1AFAP1-AS1 and sh2AFAP1-AS1), respectively. The sequences were as follows: sh1AFAP1-AS1 forward, 5'-CCG GAGCGGTCTCTGACCGAATGACTCTCGAGATCTATT CGCGTGAGACCGCTTTTTTTG-3' and reverse, 5'-AAATCTA AAAACCCGTTCTACGCGAATGACTCTCGAGATCATT TTTCGCTGAGACCGCTT-3'; sh1AFAP1-AS2 forward, 5'-CCG GACACCACTACAAGGTTGACGATCTGACCTTCT TGGGATTGTTGTTTTTTTGT-3' and reverse, 5'-AAATCTA AAAACCCAAATCCACAAAGGTGACGATCTGACCCT TCTTGGATTTGTTGT-3'. The shNC sequences were as follows: Forward, 5'-CCGGTTTCTCGGACCGTCTCAGT CTCGAGACGTGACACGTTGAGAAAATTTTTG-3' and reverse, 5'-AAATCTA AAAACCGTTCTCGGACCGTCTCAGT CTCGAGACGTGACACGTTGAGAAATTTTTT-3'; and used as negative control. These aforementioned recombinant lentiviruses vectors were purchased from Huayueyang Biotechnology, Co., Ltd. (Beijing, China). BGC-823 and SGC-7901 cells were transfected (Lipofectamine™ 3000; Thermo Fisher Scientific, Inc.) with sh1AFAP1-AS1, sh2AFAP1-AS1 or shNC according to the manufacturer's protocol. RNA amplification was performed as previously described (15). GAPDH was used to normalize mRNA expression levels. The 2^ΔΔCq method was performed as previously described (16). Sequences of the insertion vectors and primers are presented in Table I.

Cell proliferation assay. Cell Counting kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used to determine cell viability. BGC-823 and SGC-7901 cells were plated in a 96-well plate with a density of 5x10^3 cells/well. Following transfection and culturing for 12 h, CCK-8 reagents were added to each well at various time points (0, 24, 48 and 72 h), and further incubated at 37°C for 2 h. The viable cells were evaluated by detection of absorbance (450 nm) using a Multiskan™ spectrophotometer (Thermo Fisher Scientific, Inc.).

Scratch migration assays. At 24 h after transfection with sh1AFAP1-AS1 or shNC, BGC-823 and SGC-7901 cells were seeded in 24-well plates (1x10^5 cells/well), respectively. Once cells reach 90% confluence, a 200 µl pipette tip was used to produce a wound line on the cell monolayer. Images were captured at 0 and 48 h by inverted light microscopy to determine the rate of gap closure. The distance between two edges was evaluated by ImageJ v1.8.0 software (National Institutes of Health, Bethesda, MD, USA).

Matrigel invasion assay. The invasion ability of GC cells was determined by Matrigel assay. Cells were plated in 24-well plates (1x10^5 cells/well) on the top chamber with 8 µm pore size and Matrigel-coated chambers. Serum-free DMEM was added to the top chamber. The bottom chamber contained 700 µl DMEM with 20% FBS. After 24 h, cells on the upper surface were removed, while cells attached to the bottom membranes were fixed in 4% paraformaldehyde (30 min at room temperature) and stained with 0.1% crystal violet (10 min at room temperature). Images of the Matrigel assays were captured by light microscopy and the number of invasive cells was evaluated by determining the absorbance at 570 nm.

Tumorigenicity assays in nude mice. The animal experiments were approved by the Ethics Committee of The Second Xiangya Hospital of Central South University. All experiments were performed according to the National Institutes of Health animal care guidelines. Male BALB/c nude mice (4-6 weeks old; 15-20 g) were purchased from the Animal Laboratory of Central South University (Changsha, China) and randomly separated into the blank control group or the sh1AFAP1-AS1 group (3 mice per group). All mice were maintained under specific pathogen-free conditions (28°C, 10 h light and 14 h darkness) and ad libitum intake of water and food. BGC-823 or SGC-7901 cells (2x10^6 cells/mouse) were stably transfected with sh1AFAP1-AS1 or shNC vectors were injected subcutaneously into the right axilla (200 µl). All nude mice were sacrificed 42 days following tumor implantation, xenografts were dissected and tumor volume and weight were assessed. Tumor volume was determined using the following formula: Volume=0.5 x a x b^2, where a is the longest diameter and b is the transverse diameter.
 Statistical analysis. All experiments were repeated ≥3 times. Statistical analyses were performed using SPSS version 17.0 software (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). Differences between groups were analyzed using the χ² test, Fisher’s exact test, the Student’s t-test or one-way analysis of variance (Kruskal-Wallis methods for the unequal variances). Multivariate survival analysis was performed using the multivariate Cox regression test. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression level of AFAP1-AS1 and clinicopathological characteristics in GC. RT-qPCR was performed in order to detect AFAP1-AS1 expression level in 10 pairs of GC tissues and adjacent normal tissues. The results of RT-qPCR demonstrated that the expression level of AFAP1-AS1 was markedly upregulated in GC tissues in comparison with adjacent normal tissues (Fig. 1A; P<0.05). This indicated that AFAP1-AS1 may act as a tumor promoter in GC progression. To further investigate the association between AFAP1-AS1 expression level and clinicopathological parameters an additional 56 GC tissues were collected. Therefore, the analysis was performed using 66 cases of GC tissues. The higher expression level group (n=33) and lower expression level group (n=33) were categorized according to the median value of AFAP1-AS1 expression level (Fig. 1B). To identify the clinical significance of AFAP1-AS1 in GC, clinicopathological characteristics of patients including age, sex distribution, family history, ulceration, tumor size, clinical stages and differentiation were evaluated. The results demonstrated that a higher expression level of AFAP1-AS1 was significantly associated with tumor size (<2 vs. ≥2 cm; P=0.046), clinical stage (I-II stage vs. III-IV stage; P=0.001)
Table II. Association between AFAP1-AS1 expression level and clinicopathological characteristics of patients.

| Factors          | AFAP1-AS1 expression | Number | χ²   | P-value |
|------------------|----------------------|--------|------|---------|
|                  |                      | High (n=33) | Low (n=33) |       |
| Age, years       |                      |        |      |         |
| <60              |                      | 28     | 12   | 0.092   | 0.319 |
| ≥60              |                      | 38     | 17   |         |       |
| Sex distribution |                      |        |      |         |
| Female           |                      | 21     | 8    | 1.746   | 0.186 |
| Male             |                      | 45     | 20   |         |       |
| Family history   |                      |        |      |         |
| Positive         |                      | 25     | 10   | 1.610   | 0.205 |
| Negative         |                      | 41     | 23   |         |       |
| Ulceration       |                      |        |      |         |
| Positive         |                      | 24     | 11   | 0.262   | 0.609 |
| Negative         |                      | 42     | 22   |         |       |
| Tumor size, cm   |                      |        |      |         |
| <2               |                      | 38     | 15   | 3.970   | 0.046 |
| ≥2               |                      | 28     | 10   |         |       |
| Clinical stage   |                      |        |      |         |
| I-II             |                      | 41     | 14   | 10.882  | 0.001 |
| III-IV           |                      | 25     | 19   |         |       |
| Differentiation  |                      |        |      |         |
| Well             |                      | 22     | 5    | 11.643  | 0.003 |
| Moderate         |                      | 27     | 12   |         |       |
| Poor             |                      | 17     | 4    |         |       |

AFAP1-AS1, AFAP1-antisense RNA 1; n, number.

Table III. Multivariate analyses for overall survival by Cox regression test.

| Factors          | Wald P-value | RR 95% CI |
|------------------|--------------|-----------|
| Age              | 0.057        | 1.134     | 0.403    | 3.189 |
| Sex distribution | 0.177        | 1.262     | 0.427    | 3.731 |
| Family history   | 0.268        | 1.323     | 0.459    | 3.816 |
| Ulceration       | 3.236        | 0.325     | 0.095    | 1.106 |
| Tumor size       | 0.606        | 0.652     | 0.222    | 1.913 |
| Clinical stage   | 4.627        | 3.715     | 1.124    | 12.283 |
| Differentiation  | 4.144        | 0.370     | 0.142    | 0.964 |
| AFAP1-AS1 expression | 5.644     | 12.297    | 1.551    | 97.474 |

*P<0.05 was considered to indicate a statistically significant difference. Wald represents the χ² value. A higher Wald value indicates a greater difference between the two groups. AFAP1-AS1, AFAP1-antisense RNA 1; CI, confidence interval.

and tumor differentiation (P=0.003), whereas no significant association was identified with the other parameters including, age, sex distribution, family history and ulceration (Table II). These results suggested that AFAP1-AS1 may serve a vital role in the development of GC.

**AFAP1-AS1 expression level in predicting prognosis of patients with GC.** As AFAP1-AS1 expression was significantly associated with GC progression, the present study hypothesized that a high expression level of AFAP1-AS1 may affect the prognosis of patients with GC. To further investigate the prognostic significance of AFAP1-AS1 expression, univariate and multivariate analyses were performed to identify the risk factors. Multivariate analysis revealed that AFAP1-AS1 expression (RR 95% CI, 1.551-97.474; P=0.018), clinical stage (RR 95% CI, 1.124-12.283; P=0.031) and tumor differentiation (RR 95% CI, 0.142-0.964; P=0.042) were independently associated with overall survival, whereas sex distribution, family history and ulceration were not (Table III).

**Downregulation of AFAP1-AS1 inhibits GC cell proliferation ability.** In order to further investigate the role of AFAP1-AS1 in GC, GES-1 human gastric epithelial cell line and MGC-803, BGC-823 and SGC-7901 gastric cancer cell lines were used. Firstly, RT-qPCR was performed to detect the AFAP1-AS1 expression level in gastric epithelial cell and GC cell lines. The results revealed that AFAP1-AS1 was expressed at higher levels in the GC cell lines compared with in GES-1 cells, and SGC-7901 and BGC-823 cells exhibited higher expression levels (Fig. 2A). Based on the preliminary data, the present study selected SGC-7901 and BGC-823 cells for further investigation in vitro. sh1AFAP1-AS1, sh2AFAP1-AS1 or shNC vectors were transfected into SGC-7901 and BGC-823 cells. RT-qPCR results demonstrated that the first shRNA sequence had the greatest inhibitory effect. Therefore, the sh1AFAP1-AS1 vector was selected for further study (Fig. 2B). In order to elucidate whether AFAP1-AS1 affected GC cell proliferation, CCK-8 colorimetry was performed to determine cell viability. The results of the CCK-8 assay revealed that proliferation rates in the sh1AFAP1-AS1 transfected group were significantly lower compared with the control group (shNC) in SGC-7901 and BGC-823 cells at 48 and 74 h (Fig. 2C). This indicated that downregulation AFAP1-AS1 expression markedly inhibited GC cell viability.

**Downregulation of AFAP1-AS1 inhibits GC cell migration and invasion ability.** Migration and invasion abilities were used to evaluate the tumor biological features. A scratch assay was performed to detect the migration of GC cells. The results revealed that SGC-7901 and BGC-823 cells in the sh1AFAP1-AS1 group did not exhibit the same level of space closure as shNC groups at 48 h (Fig. 3A), indicating that the movement ability of GC cells was significantly suppressed by knockdown of AFAP1-AS1. In addition, a Matrigel assay was performed to determine the invasion ability of GC cells. As expected, AFAP1-AS1 depletion resulted in a reduction of the number of invading cells in BGC-823 and SGC-7901 cell lines (Fig. 3B). In brief, knockdown of AFAP1-AS1 negatively regulated GC cell proliferation,
migration and invasion, indicating that AFAP1-AS1 may function as a tumor promoter in GC tumorigenesis.

**Downregulation of AFAP1-AS1 suppresses tumor growth in nude mice.** Nude mice experiments were performed to confirm the effect of the knockdown of AFAP1-AS1 in GC cells in vivo. SGC-7901 and BGC-823 cells were transfected with the shAFAP1-AS1 vector or blank empty (shNC) vector, respectively. The growth curves for each group are presented in Fig. 4A. A rapid increase in tumor volumes at day 35 in the shNC group were observed, which revealed that AFAP1-AS1 knockdown cells demonstrated significantly lower tumorigenicity (P=0.031 for SGC-7901 cells; P=0.008 for BGC-823 cells). At the end of the experiment, mice were sacrificed. The mean tumor volumes of mice transfected with SGC-7901 and BGC-823 cells in the blank control group were 2,084.22±513.05 and 2,260.10±981.73 mm$^3$, which were higher compared with the shAFAP1-AS1 group (684.27±95.22 and 496.37±284.46 mm$^3$), respectively (Fig. 4B and C; P<0.05). Slower tumor growth was observed in the shAFAP1-AS1 group compared with the control group.

**Discussion**

An increasing number of studies have suggested that understanding the function of IncRNA may provide us with a better understanding of the tumor process (16-18). For the IncRNA study of gastric cancer, the gene H19 was first reported in 2012 (12). H19 overexpression increased cellular proliferation, whereas downregulation of H19 increased the rate of apoptosis, which was associated with p53 inactivation (19). Previous studies have confirmed that the dysregulation of IncRNAs participates in GC development (20,21). Liu et al (22) demonstrated that the upregulation HOX transcription antisense RNA (HOTAIR) was associated with tumor size and metastasis and represented a biomarker of poor prognosis in gastric cancer. HOTAIR depletion significantly suppressed cell viability and invasion in vitro and in vivo. Antisense RNA in the INK4 locus (23) and colon cancer-associated transcription 1 (24) have been characterized to have a growth-promotion function, whereas FOXF1 adjacent non-coding developmental regulatory RNA (25),
double homeobox A pseudogene 8 (26), metastasis associated lung adenocarcinoma transcript 1 (27) and HOXA distal transcript antisense RNA (28) have been confirmed to have metastatic-promotion functions in GC.

AFAP1-AS1, also known as LOC84740, is located on chromosome Chr4p16.1 and acts as an antisense lncRNA, which functions as a regulator of the AFAP1 gene (29). AFAP1-AS1 expression is frequently upregulated and serves a vital role in a variety of tumor types, including colorectal cancer, hepatocellular carcinoma, esophageal squamous cancer and non-small cell lung cancer (30-34). A previous study identified 186 upregulated and 294 downregulated lncRNAs that were expressed with significantly differential expression levels in GC via lncRNA microarray-analysis, including AFAP1-AS1 (14). However, the detail mechanisms of AFAP1-AS1 underlying GC progression require further investigation. The present study first detected the AFAP1-AS1 expression level in 10 pairs of GC and adjacent tissues by RT-qPCR, and a higher expression level in GC tissues compared with in adjacent tissues was revealed. This result was consistent with a previous study (7). In order to determine whether AFAP1-AS1 acts as a tumor promoter in GC, the number of GC tissue samples analyzed was increased and the association between AFAP1-AS1 expression level
and clinicopathological parameters was investigated. The results indicated that AFAP1-AS1 expression level was positively correlated with GC development. A high AFAP1-AS1 expression level was associated with poor prognosis in GC patients by performing multivariate Cox regression test analysis. In order to explore the effect of AFAP1-AS1 in vitro, the expression AFAP1-AS1 was downregulated in SGC-7901 and BGC-823 cells. The CCK-8 assays revealed that a low expression level of AFAP1-AS1 suppressed the proliferation of GC cells. Scratch and Matrigel assays demonstrated that downregulation AFAP1-AS1 inhibits cell migration and invasion abilities. To further explore the promotional role of AFAP1-AS1, experiments using mice were performed. The transfected SGC-7901 or BGC-823 cells were injected in nude mice subcutaneously. A total of 42 days following implantation, mice were sacrificed and the xenograft tumor was dissected. The mean volume and weight of tumors in the shNC group was greater compared with that in the shAFAP1-AS1 group. Taken together, these findings suggested that reducing AFAP1-AS1 expression level significantly inhibited GC development. However, the exact mechanisms underlying AFAP1-AS1 remain to be elucidated. In future studies, a greater number of GC tissue samples should be obtained in order to reach a more credible conclusion regarding the role of AFAP1-AS1. The target genes of AFAP1-AS1 and relevant downstream signaling pathways also require further investigation.

In conclusion, the present study demonstrated that AFAP1-AS1 expression level was markedly upregulated in GC tissues and cell lines. Downregulation of AFAP1-AS1 inhibited cell proliferation, invasion and migration in vitro and also tumor growth of GC cells in vivo. These results indicated that AFAP1-AS1 may serve as a novel prognostic marker and tumor promoter in GC, and the newly identified AFAP1-AS1 may provide a promising molecular target for anticancer therapy.
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