European Review for Medical and Pharmacological Sciences 2017; 21: 3842-3849

Increased lncRNA AFAP1-AS1 expression predicts poor prognosis and promotes malignant phenotypes in gastric cancer

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Abstract. – OBJECTIVE: The clinical significance and biological functions of long non-coding RNA AFAP1-AS1 in gastric cancer (GC) remain larger elucidated. The aim of the study is to investigate the role of lncRNA AFAP1-AS1 involved in GC progression.

PATIENTS AND METHODS: Quantitative reverse transcription-polymerase chain reaction (QRT-PCR) assay was used to evaluate the expression of lncRNA AFAP1-AS1 in GC tissues, when compared with adjacent noncancerous tissues, respectively. Associations between lncRNA AFAP1-AS1 and the clinicopathological factors in GC patients were analyzed by X^2-test. The prognostic significance was evaluated using Kaplan-Meier curve and Cox regression analysis. CCK8, flow cytometry analysis and transwell invasion assays were performed to assess cell proliferation and invasion abilities of GC.

RESULTS: In this study, we verified that lncRNA AFAP1-AS1 expression was dramatically increased in GC tissues and cells, compared with noncancerous gastric tissues and control cells. The higher lncRNA AFAP1-AS1 expression was positively correlated with lymph node metastasis ($p = 0.001$), TNM stage ($p = 0.006$) and worse overall survival (OS) time in GC patients. Multivariate Cox analysis suggested that lymph node metastasis (Hazard ratio, HR = 2.966, $p = 0.001$), TNM stage (HR = 2.855, $p = 0.001$), and lncRNA AFAP1-AS1 expression levels (HR = 3.315, $p = 0.001$) were independent prognostic factors of OS in GC patients. Knockdown of lncRNA AFAP1-AS1 significantly inhibited the cell proliferation and cell cycle progression. Moreover, reduced lncRNA AFAP1-AS1 also inhibited the cell invasion ability via regulating cell epithelial-mesenchymal transition (EMT) phenomenon in GC.

CONCLUSIONS: These results demonstrated that lncRNA AFAP1-AS1 may be a potential therapeutic target for GC.

Key Words:
Long non-coding RNA, Gastric cancer, AFAP1-AS1, Tumor prognosis.

Introduction

Gastric cancer (GC) is one of the most common malignant tumors and ranks as the second leading cause of cancer mortality worldwide[1]. In the past decades, the development of clinical treatment leads to the decrease in the incidence and mortality of GC patients. However, the 5-year survival rate of advancing gastric cancer patients remains largely dissatisfied[2,3]. Therefore, understanding the molecular mechanisms involved in GC development and explored novel biomarkers and potential therapeutic targets for GC are urgently needed. Long non-coding RNAs (LncRNAs) are more than 200 nt in length. Some studies have demonstrated that lncRNAs play crucial roles in tumor process including regulating cell growth, apoptosis, differentiation, invasion, and metastasis at the transcriptional and post-transcriptional levels[4]. Some LncRNAs including GHET1, MALAT1, TINCR, ANRIL, GACAT3, H19 and MEG3 exhibit their biological roles in GC progression by acting as oncogenes or tumor suppressors[5-7]. Long non-coding RNA AFAP1-AS1 was overexpressed and firstly found in the Barrett's esophagus and esophageal adenocarcinoma[8]. Higher AFAP1-AS1 expression was associated with tumor size, TNM stage, vascular invasion, and poor prognosis. Moreover, higher lncRNA AFAP1-AS1 expression promoted cell proliferation and tumor metastasis of hepatocellular carcinoma[9]. The lncRNA AFAP1-AS1

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depletion resulted in the inhibition of colorectal cancer (CRC) cell proliferation, colony formation and induced G0/G1 cell cycle arrest in CRC cells[10]. However, there is no relative evidence for lncRNA AFAP1-AS1 whether affecting gastric cancer progression. In the current study, we showed that LncRNA AFAP1-AS1 was dramatically increased in GC tissues and higher LncRNA AFAP1-AS1 predicted a shorter survival time for patients. Furthermore, LncRNA AFAP1-AS1 silencing suppressed the GC cell proliferation, cell invasion and cell EMT process. These results demonstrated that lncRNA AFAP1-AS1 may be a novel biomarker for prognosis and therapeutic target for GC.

Patients and Methods

Patients and Tissue Samples

91 primary gastric cancer tissues and their paired adjacent normal tissues were collected from patients who underwent surgery resection at the Department of Medical Oncology, the First Affiliated Hospital of Nanjing Medical University (Nanjing, China). The fresh GC tissue samples were frozen in liquid nitrogen and stored at -80°C. GC patients were confirmed as gastric adenocarcinoma by two experienced pathologists. These patients had no any chemotherapy and radiotherapy before surgery. Tumor staging was identified according to the sixth edition of tumor-node-metastasis (TNM) classification for esophageal carcinoma (UICC, 2002). Written informed consents were collected from all patients and the study was approved by Ethics Committee of the First Affiliated Hospital of Nanjing Medical University.

Cell Lines and Cell Transfection

Four human gastric cancer cell lines (AGS, BGC-823, MGC-803 and SGC-7901) and an immortalized human gastric epithelial mucosa cell line (GES-1) were cultured using RPMI-1640 medium (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) at 37°C with 5% CO_2. The two siRNAs oligos (si-AFAP1-AS1-1 and si-AFAP1-AS1-2) and si-negative control were synthesized by GenePharma (Shanghai, China). For cell transfection, 1×10^5 cell/well were seeded in 6-well plates and cells transfection was performed using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA). The siRNAs sequences were as follows: si-AFAP1-AS1-1: 5'-GGGCTTCAATTTACAAGCATT-3', and si-AFAP1-AS1-2:5'-CCTATCTGGTCAACACGTATT-3'.

RNA Isolation and Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from tissue specimens and cells by using Trizol (Invitrogen, Carlsbad, CA, USA). The reverse transcription reactions were carried out using Transcriptor First Strand cDNA Synthesis Kit (TaKaRa, Dalian, Liaoning, China). Real-time PCR was performed using SYBR Green SuperMix (TaKaRa, Dalian, Liaoning, China) by ABI7900HT fast Real-time PCR system (Applied Biosystems, Foster City, CA, USA). GAPDH was used as an internal control. The mRNA expression levels were analyzed by $2^{-\Delta\Delta Ct}$ method. The primers were lncRNA AFAP1-AS1, forward: 5'-TCGCTCAATGGAGTGACGGCA-3', and reverse: 5'-CGGCTGAGACCGCTGAGAACTT-3'. GAPDH, forward: 5'-GTCAACGGATTTGGTCTGTATT-3' and reverse: 5'-AGTCTTCTGGGTGGCAGTGAT-3'. All results were presented as the mean ± standard deviation of three independent experiments.

CCK-8 Assay

Cell Counting Kit-8 (CCK-8) (Dojindo, Kunamoto, Japan) was applied to determine the cells proliferation ability according to the manufacturer's instructions. The 1×10^5 cell/well cells were seeded in triplicate in 96-well plates and cultured for 24 h. Then, cells transfected with si-AFAP1-AS1-1, si-AFAP1-AS1-2 and si-NC were detected at 1, 2, 3, 4 and 5 days, and the absorbance was measured at 450 nm on a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The results were derived from triplicate samples and are presented as mean ± SD.

Cell Invasion Assay

Transwell assays were performed to assess the invasive cell ability of GC cells. Briefly, after 24 h transfection with si-AFAP1-AS1-1, si-AFAP1-AS1-2 and si-NC, 1×10^5 cells supplemented with 200 μL serum-free DMEM medium were added pre-coated with 100 mg matrigel in the upper chambers (BD Biosciences, San Jose, CA, USA). 500 μL medium containing 10% fetal bovine serum (FBS) was added to the lower chambers. After incubation at 37°C for another 24 h, cells in the upper chambers were removed and cells in the lower chambers were fixed; then, they were stained and visualized under an Olympus microscope (Tokyo, Japan).

Western Blot Assays

Cells were lysed using RIPA lysis buffer (Beyotime, Shanghai, China) on ice. The pro-

tein concentration was evaluated using a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). The extract equivalent to 40 ng of total protein was separated by a 10% using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then followed by transfer to polyvinylidene fluoride (PVDF) membrane. The membranes were blocked in 5% non-fat milk diluted in tris buffered saline tween (TBST) and incubated with the indicated specific antibody with e-cadherin (dilution rates of 1:1000, Cell Signaling Technologies, Danvers, MA, USA), vimentin (dilution rates of 1:1000, Cell Signaling Technologies, Danvers, MA, USA) and GAPDH (dilution rates of 1:1000, Cell Signaling Technologies, Danvers, MA, USA). After incubation with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature, proteins were detected using a Chemi Doc XRS imaging system and Quantity One analysis software (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical Analysis

Each experiment was repeated at least three times. The data was performed using SPSS17.0 (IBM Corp., Armonk, NY, USA). Statistical analysis was presented as mean ± SD and two way ANOVA was used for the analysis for difference between groups when > 2 groups were present; otherwise, a Student's *t* test was used. A p-value < 0.05 was considered statistically significant.

Results

Expression of lncRNA AFAP1-AS1 is Significantly Increased in GC Tissues and Cells

In this study, we determined the lncRNA AFAP1-AS1 expression levels by qRT-PCR from 91 GC tissues and paired corresponding adjacent non-cancerous tissues. The results found that GC tissues samples had higher lncRNA AFAP1-AS1 expression compared with adjacent non-cancerous tissues (Figure 1A). Meanwhile, the lncRNA AFAP1-AS1 expression was positively correlated with lymph node metastasis (p = 0.001) and TNM stage (p = 0.006) in gastric cancer patients (Figure 1B-C, Table I). No association with other factors such as, age (p = 0.190), sex (p = 0.876), histological differentiation (p = 0.916), etc. (Table I). Furthermore, mul-

Table I. Association between lncRNA AFAP1-AS1 expression and clinicopathologic factors in GC patients was determined by X^2 test.

Clinicopathological factors	GC patients (n=91)	AFAP1-AS1 expression level		X^2-test, p-value
		Lower (48)	Higher (43)	
Age				0.190
≤ 55	40	18	22	
> 55	51	30	21	
Sex				0.876
Male	60	32	28	
Female	31	16	15	
Lymph node metastasis				0.001a
Negative	48	33	15	
Positive	43	15	28	
Histological differentiation				0.916
High, middle	63	33	30	
Poor	28	15	13	
Tumor size				
< 5 cm	66	38	28	0.834
> 5 cm	25	10	15	
Local invasion				0.275
T1, T2	52	30	22	
T3, T4	39	18	21	
TNM stage				0.006[a]
I-II	49	33	16	
III-IV	42	15	27	

[a]p-value <0.05 was considered statistically significant.

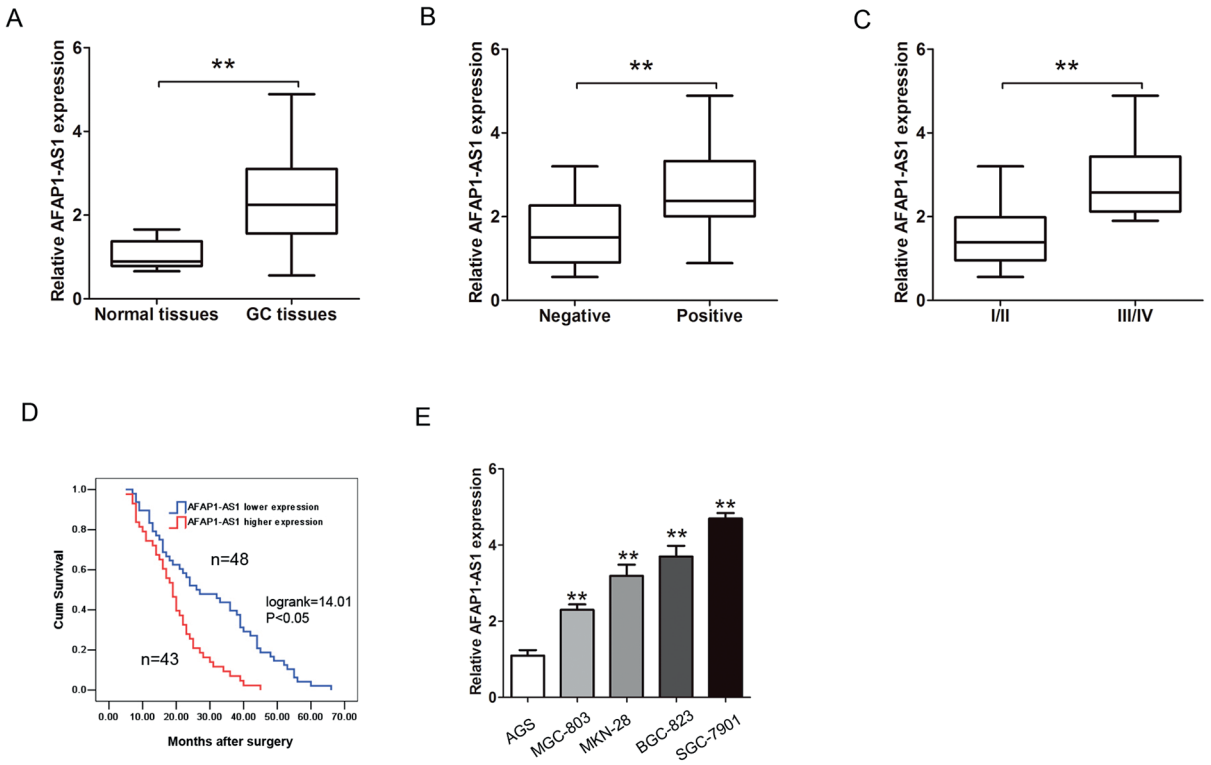

Figure 1. LncRNA AFAP1-AS1 expression was elevated in gastric cancer tissues and cell lines. ***(A)*** LncRNA AFAP1-AS1 expression was increased in gastric cancer tissues compared with paired corresponding adjacent non-cancerous tissues. ***(B-C)*** the association between lncRNA AFAP1-AS1 expression and lymph node metastasis or TNM stage was shown. ***(D)*** patients who had higher expression of lncRNA AFAP1-AS1 predicted a poor prognosis when compared with lower LncRNA AFAP1-AS1 expression (log rank test). ***(E)*** the LncRNA AFAP1-AS1 expression was increased in gastric cancer cells compared with an immortalized human gastric epithelial mucosa cell line (GES-1). All results were presented as the mean ± standard deviation of three independent experiments, $^{**}p<0.05$.

tivariate Cox regression analysis indicated that the lymph node metastasis (hazard ratio HR = 2.966, p = 0.001), TNM stage (HR = 2.855, p = 0.001), and lncRNA AFAP1-AS1 expression levels (HR = 3.315, p = 0.001) were independent prognostic factors for OS time in gastric cancer patients (Table II). Kaplan-Meier survival curve analysis revealed that patients with higher lncRNA AFAP1-AS1 expression levels showed poor survival probability compared with lower lncRNA AFAP1-AS1 expression levels (Figure 1D, $p < 0.05$). Moreover, we also examined the lncRNA AFAP1-AS1 expression levels in four human gastric cancer cell lines (AGS, BGC-823, MGC-803 and SGC-7901) and an immortalized human gastric epithelial mucosa cell line (GES-1). The results showed that lncRNA AFAP1-AS1 expression was increased in GC cells, compared with the control cells (Figure 1E). Thus, we demonstrated the lncRNA AFAP1-AS1 was increased in GC tissues and cells, acting as a predictor for prognosis of GC patients.

Table II. Multivariate analysis of over survival (OS) for prognosis in GC patients (n=91).

Variables	HR	95%CI	*p*-value
Lymph node metastasis	2.966	1.366-5.338	0.001**
TNM stage	2.855	1.143-5.184	0.001**
lncRNA AFAP1-AS1	3.315	1.553-5.899	0.001**

**, p-value <0.05 was considered statistically significant, CI, confidence intervals.

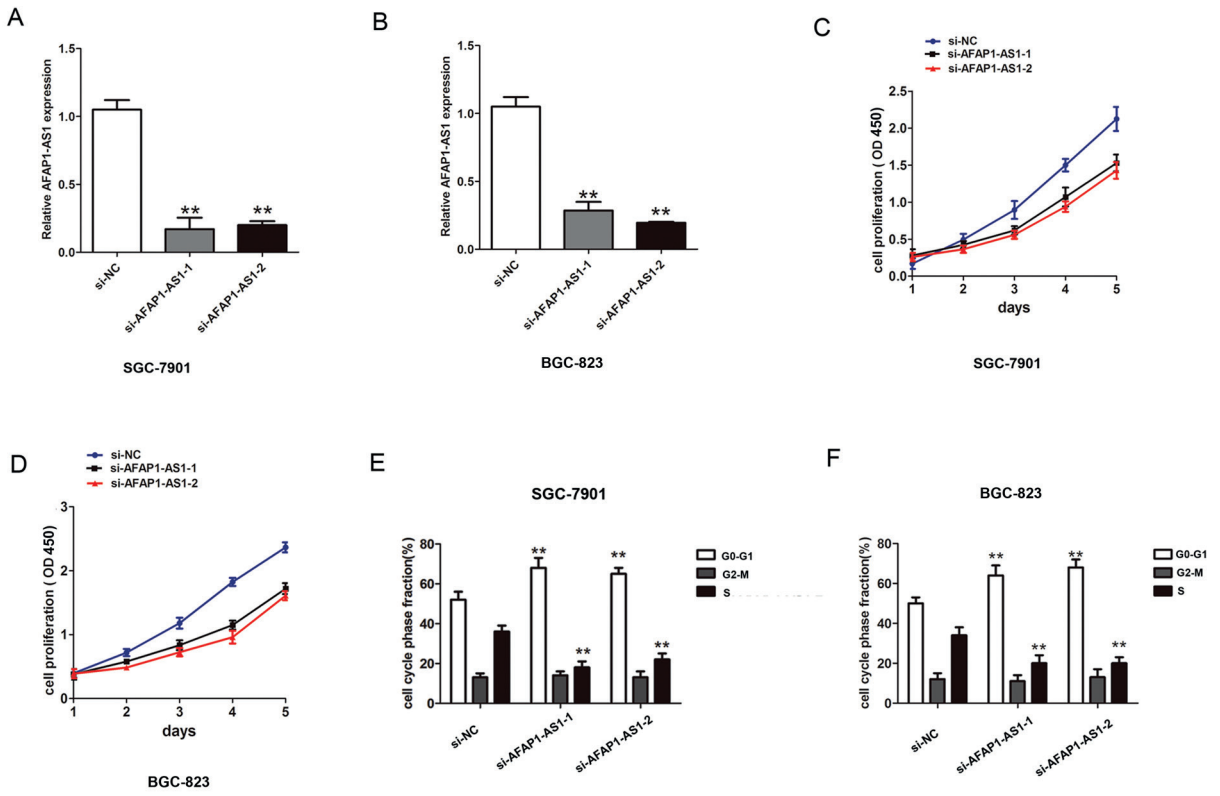

Figure 2. Knockdown of LncRNA AFAP1-AS1 suppressed the GC cell proliferation and cell cycle progression. ***(A-B)*** LncRNA AFAP1-AS1 expression levels were determined by qRT-PCR assays after transfected with si-NC, si-AFAP1-AS1-1 and si-AFAP1-AS1-2 into SGC-7901 and BGC-823 cells. ***(C-D)*** cell proliferation ability was determined by CCK8 assays after transfected with si-NC, si-AFAP1-AS1-1and si-AFAP1-AS1-2 into SGC-7901 and BGC-823 cells. ***(E-F)*** cell cycle was determined by flow cytometry analysis after transfected with si-NC, si-AFAP1-AS1-1and si-AFAP1-AS1-2 into SGC-7901 and BGC-823 cells. All results were presented as the mean ± standard deviation of three independent experiments, ** $p < 0.05$.

The lncRNA AFAP1-AS1 Enhances Cell Proliferation and Cell Cycle Progression

To further confirm lncRNA AFAP1-AS1 expression whether affected the cell proliferation, we performed the CCK8 and flow cytometry analysis. The results demonstrated that si-AFAP1-AS1-1 and si-AFAP1-AS1-2 oligos significantly inhibited the lncRNA AFAP1-AS1 expression levels in SGC-7901 or BGC-823 cells, compared with the si-NC group (Figure 2A-2B). As shown by CCK8 assays, our results suggested that knockdown of lncRNA AFAP1-AS1 in SGC-7901 or BGC-823 cells significantly inhibited cell proliferation, compared with si-NC group (Figure 2C-D). The flow cytometry analysis results showed that knockdown of lncRNA AFAP1-AS1 in SGC-7901 or BGC-823 cells significantly increased the G1 phase cell proportion, but reduced the S-phase cell proportion, compared with si-NC group (Figure 2E-F). These data suggested that reduced lncRNA AFAP1-AS1 expression in GC suppressed cell proliferation and cell cycle progress.

The lncRNA AFAP1-AS1 Enhances Cell Invasion and Regulates EMT Process

Furthermore, we examined the cell invasion ability after knockdown of lncRNA AFAP1-AS1 by performing transwell cell invasion assays. The results suggested that knockdown of lncRNA AFAP1-AS1 significantly inhibited cell invasion ability and reduced the invasive cell number when compared with si-NC group in SGC-7901 or BGC-823 cells (Figure 3A-D). Also, we explored the effects of lncRNA AFAP1-AS1 expression on GC cell EMT process. Here, we found that the EMT maker E-cadherin expression was dramatically up-regulated, but vimentin expression was down-regulated when lncRNA AFAP1-AS1 was knocked down in SGC-7901 cells or BGC-823 cells (Figure 3E-F). These above studies showed that lncRNA AFAP1-AS1 could

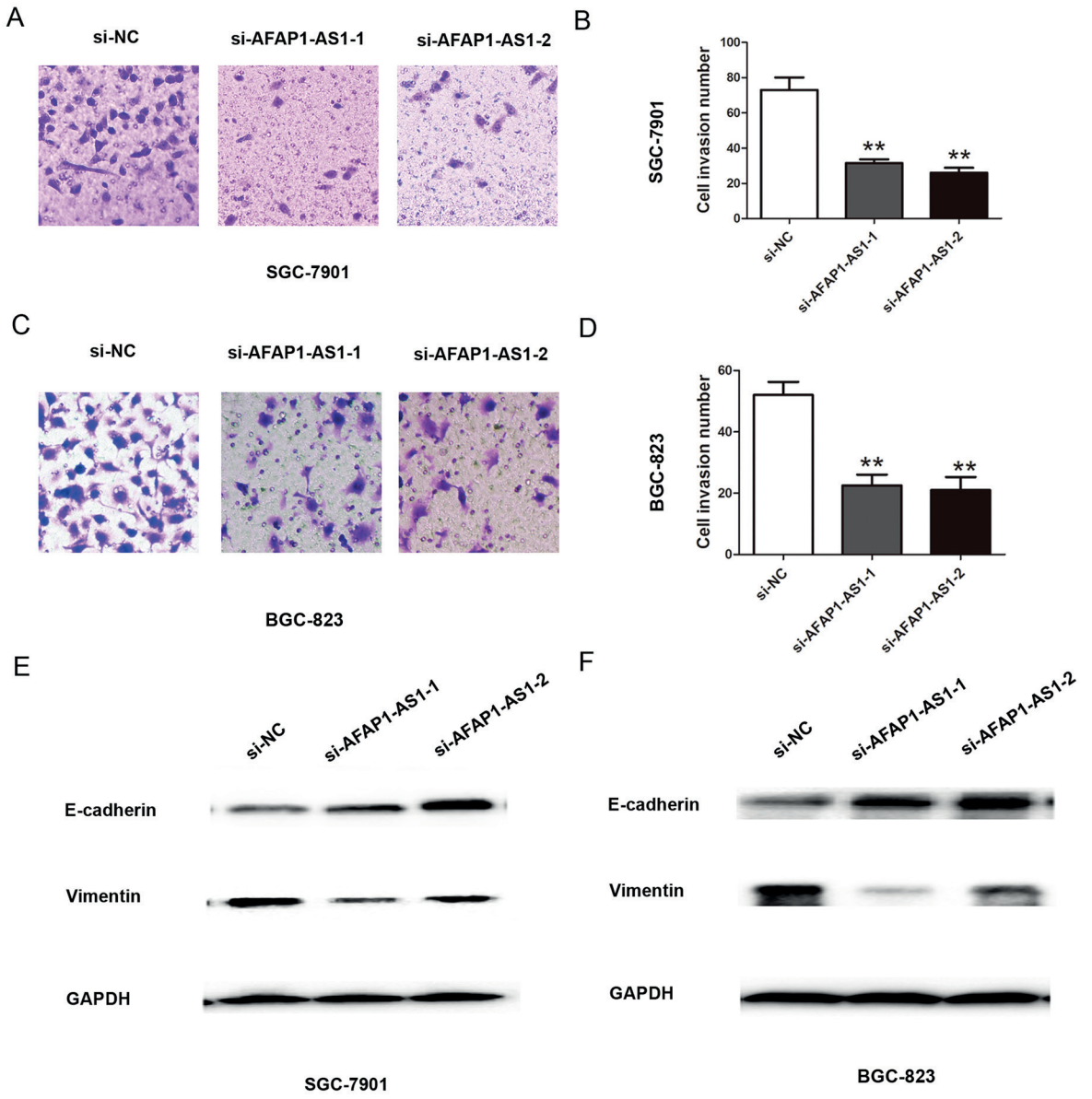

Figure 3. Downregulation of lncRNA AFAP1-AS1 inhibited cell invasion and EMT process. ***(A-B)*** cell invasion ability was determined by transwell assays after transfected with si-NC, si-AFAP1-AS1-1 and si-AFAP1-AS1-2 into SGC-7901. ***(C-D)*** cell invasion ability was determined by transwell assays after transfected with si-NC, si-AFAP1-AS1-1 and si-AFAP1-AS1-2 into BGC-823 cells. ***(E-F)*** the protein expression of E-cadherin and vimentin were determined by Western-blot analysis after transfected with si-NC, si-AFAP1-AS1-1 and si-AFAP1-AS1-2 into SGC-7901 or BGC-823 cells. All results were presented as the mean ± standard deviation of three independent experiments, $^{**}p < 0.05$.

promote cell invasion and enhance the EMT process in GC cells.

Discussion

Accumulating studies revealed that lncRNAs play important roles in a wide range of biological processes. Recent researches have revealed that lncRNAs are often aberrantly expressed in gastric cancer. For example, lncRNA Sox2 overlapping transcript (Sox2ot) in GC tissues served as a poor prognostic biomarker and acted as oncogene to promote cell proliferation and invasion[11]. Linc00261 was found to suppress gastric cancer progression by promoting the degradation

of slug via enhancing the interaction between GSK3β and slug[12]. LncRNA-RMRP promoted carcinogenesis by acting as a miR-206 sponge and was used as a novel biomarker for gastric cancer[13]. MALAT1 could promote cell proliferation, cell cycle progression, migration and invasion, and induce apoptosis in gastric cancer[14]. In the study, we found that LncRNA AFAP1-AS1 expression was higher and positively correlated with lymph node metastasis, and advanced tumor stage, as well as poor prognostic outcome in GC patients. A previous work showed that upregulation of long non-coding RNA AFAP1-AS1 was found to be associated with the poor prognosis of NSCLC patients[15]. Upregulated of long non-coding RNA AFAP1-AS1 expression levels were significantly associated with poor prognosis of nasopharyngeal carcinoma and promoted cancer cell metastasis via regulation of actin filament integrity[16]. Higher expression of AFAP1-AS1 predicted a poor survival time and short-term recurrence in pancreatic ductal adenocarcinoma patients[17]. Our results showed that LncRNA AFAP1-AS1 silencing decreased GC cell proliferation. Moreover, knockdown of lncRNA AFAP1-AS1 significantly increased the G1 phase cell proportion, but reduced the S-phase cell proportion in SGC-7901 or BGC-823 cells. Recent findings exhibited that AFAP1-AS1 was significantly elevated in colorectal carcinoma (CRC) and AFAP1-AS1 knockdown inhibited tumor metastasis-associated genes expression in terms of EMT[18]. AFAP1-AS1 promoted cell proliferation and invasion abilities via upregulation of the RhoA/Rac2 signaling[19]. Knockdown of AFAP1-AS1 significantly inhibited the lung cancer cell invasive and migration capability[20]. Similarly, our studies showed lncRNA AFAP1-AS1 promoted GC cell EMT process by inhibiting E-cadherin expression levels, but upregulating the vimentin expression in GC cells.

Conclusions

We found that lncRNA AFAP1-AS1 was upregulated in GC and play as an important biomarker of prognosis in GC patients. Therefore, lncRNA AFAP1-AS1 may be a biomarker for prognosis and a therapeutic target of GC.

Conflict of interest

The authors declare no conflicts of interest.

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