Original Article

High expression of IncRNA AFAP1-AS1 promotes cell proliferation and invasion by inducing epithelial-to-mesenchymal transition in gastric cancer

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Abstract: Objectives: The lncRNA AFAP1-AS1 was reported to be involved in the progression of many types of tumors. However, the expression and molecular mechanism of AFAP1-AS1 in gastric cancer remains unclear. Methods: The relative expression level of lncRNA AFAP1-AS1 was determined by qRT-PCR in a total of 87 patients with gastric cancer and four gastric cancer cell lines. We inhibited AFAP1-AS1 expression by transfecting AFAP1-AS1 specific small interfering RNA (si-AFAP1-AS1). Cell proliferation was determined by using MTT assay. Cell apoptosis was determined by using Flow cytometry. Cell invasion was determined by using transwell invasion assay. Furthermore, western blot was used to exploring the effected of AFAP1-AS1 on epithelial-to-mesenchymal transition (EMT) process in gastric cancer. Results: We found that lncRNA AFAP1-AS1 expression was upregulated in gastric cancer tissues and cell lines compared with matched adjacent non-tumor tissues and normal gastric epithelial cell line GES-1. High expression level of AFAP1-AS1 correlated with lymph node invasion, distant metastasis, advanced TNM stage, and poor prognosis. SiRNA of AFAP1-AS1 efficiently downregulated the expression of AFAP1-AS1 in gastric cancer cell lines. Knockdown of AFAP1-AS1 inhibited the proliferation and invasion ability of gastric cancer cells in vitro. Furthermore, western blot showed that suppressed AFAP1-AS1 expression in gastric cancer cells resulted in increased E-cadherin protein expression and decreased N-cadherin and Vimentin protein expression. Conclusion: Our study suggested that AFAP1-AS1 play oncogenic roles and could be used as a therapeutic target for treating human gastric cancer.

Keywords: AFAP1-AS1, LncRNA, gastric cancer, epithelial-to-mesenchymal transition, progression

Introduction

Gastric cancer is the second most common carcinoma of human in the world, which contributes to 10% newly diagnosed cases every year and high mortality worldwide [1]. Surgery and followed chemotherapy bring about a clinical response at 20% to 35%, and intensive research has validated some of the molecular mechanism associated with gastric cancer bio-chemical functional events [2, 3]. However, the total mechanism of gastric cancer initiation and progress leaves researchers largely unknown.

The long non-coding RNAs (lncRNAs) are a class of RNA transcripts over 200 nucleotides in length and possess no protein-coding capacity [4]. Recent studies showed that IncRNAs play important roles in cellular development, differentiation, and many other biological processes [5-7]. Dysregulation of IncRNAs are involved in various types of cancer, including gastric cancer. For example, Xia et al revealed that lncRNA MALAT1 could function as an oncogene in gastric cancer, and high MALAT1 level could serve as a potential biomarker for the distant metastasis of gastric cancer [8]. Li et al showed that IncRNA CASC2 suppressed the proliferation of gastric cancer cells by regulating the MAPK signaling pathway [9]. Chen et al showed that IncRNA XIST regulated gastric cancer progression by acting as a molecular sponge of miR-101 to modulate EZH2 expression [10]. However, IncRNA AFAP1-AS1 (actin filament associated protein 1 antisense
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RNA1) expression in gastric cancer and the underlying mechanisms of its effects remains unclear.

In the present study, we explored the expression and clinical significance of IncRNA AFAP1-AS1 in gastric cancer. By knockdown AFAP1-AS1 expression, we investigated the function of AFAP1-AS1 on gastric cancer cell proliferation, apoptosis and invasion. In addition, the effect of AFAP1-AS1 on epithelial-to-mesenchymal transition (EMT) process was explored.

Materials and methods

Patients and sample collection

Pairs of gastric cancer tissues and adjacent non-tumor tissues were available from 87 patients undergoing surgical procedures at The Second People’s Hospital of Jiaozuo, between January 2009 and January 2011. Both tumors and adjacent non-tumor tissues were submitted to histological analysis for diagnostic confirmation. After resection, all samples were immersed immediately in RNA later solution (Ambion) overnight, and stored at -80°C in order to avoid degradation of RNA. Prior to the use of these clinical materials for research purposes, written consents from all patients and approval of The Second People’s Hospital of Jiaozuo Ethic Review Committees were obtained.

Cell culture and transfection

Human gastric cancer cell lines HGC-27, SGC-7901, MKN-45, BGC-823, and the normal gastric epithelial cell line GES-1 were obtained from American type culture collection (ATCC). All cell lines were cultured in RPMI-1640 (Gibco) with 10% fetal bovine serum (FBS) (HyClone) as well as 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen). Cells were maintained in a humidified incubator in the presence of 5% CO₂ at 37°C.

In order to suppress the expression of AFAP1-AS1 in gastric cancer cell line, siRNAs against AFAP1-AS1 were designed and synthesized by Ribobio. The sequences of two siRNAs against AFAP1-AS1 were designed as follows: 5'-GGGCTTCAATTACAGACATT-3' (si-AFAP1-AS1-1) and 5'-CCTACTCGTCAACACGTATT-3' (si-AFAP1-AS1-2). siRNAs were transfected into gastric cancer cells by Lipofectamine 2000 (Invitrogen).

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from clinical samples and cell lines by Trizol reagent (Life Technologies). The GeneAmp RNA PCR kit (Life Technologies) was used to reverse-transcribe RNA to complementary DNA for the gene expression analysis. For qRT-PCR, three replicates of each sample were amplified in a 20 μL reaction mixture containing SYBR Green reaction mix (Qiagen) and 0.5 mM of primer, and analyzed using a Roche Light-Cycler (Roche). The primers for qRT-PCR are as follows: AFAP1-AS1, Forward 5'-TCGCTCAATGGAGTACGGCA-3'; Reverse 5'-CGCGCTAGACCGCTGAAACTT-3'; GAPDH, Forward 5'-GACTCATGACCCACAGTCCATGC-3', Reverse 5'-AGAGCCAGGGATGATGTTCTG-3'

Cell proliferation assay

Cell proliferation assay was performed using the MTT kit according to the manufacturer’s instruction (Roche). Briefly, after transfection for 24 h, 3,000 cells per well were allowed to grow in 96-well plates with five replicate wells. After 6 h of culture, as well as at 24, 48, 72 and 96 h after starting the culture, the cells were treated with 100 μg MTT by adding it to the medium. The cells were incubated at 37°C for another 4 h, then the medium was removed, and DMSO was added for 10 min to lyse the cells. Finally, the absorbance at a wave length of 490 nm was determined using a microplate reader.

Cell apoptosis assay

Transfected cells were harvested after transfection by trypsinization. After the double staining with fluorescein isothiocyanate (FITC)-Annexin V and propidium iodide was done by the FITC Annexin V Apoptosis Detection Kit (BD Biosciences) according to the manufacturer, the cells were analyzed with a flow cytometry (FACScan; BD Biosciences) equipped with a Cell Quest software (BD Biosciences).
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Cells in 100 μl of serum-free medium were added to the top chamber of 24-well plates that have been pre-added with Matrigel. The bottom well contained growth medium with 20% FBS. Transwell chambers were placed at 37°C for 48 h. Cells in chamber were fixed with methanol for 30 min and then staining with Giemsa for 15-30 min. Invaded cells were finally observed under a microscope and the number was counted with randomly nine field for each experiment.

**Western blot**

Cells were lysed in lysis buffer in the presence of Aprotinin, Leupeptin, Phenylmethanesulfonyl fluoride (PMSF) (Sigma) and phosphatase inhibitor cocktails II and III (Sigma). Proteins were measured by Bradford method. Then, 50 mg of total protein extracts was fractionated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene diuoride membranes (GE Healthcare). The membrane was incubated with the following primary antibodies: anti E-cadherin, anti-N-cadherin, anti-Vimentin and anti-GAPDH antibody (Santa Cruz). Binding of the primary antibody was detected using an enhanced chemiluminescence kit (ECL Amersham).

**Statistical analyses**

All statistical analyses were performed using the SPSS 17.0 software package. The significance of differences between groups was estimated by Student’s t-test and chi-square test. The results are reported as the means ± SD. P<0.05 was considered to be statistically significant.

**Results**

*IncRNA AFAP1-AS1 expression is upregulated in gastric cancer tissues and cell lines*

To explore whether AFAP1-AS1 is associated with gastric cancer progression, we determined AFAP1-AS1 expression in gastric cancer tissues. We found that the expression of AFAP1-AS1 was significantly upregulated in gastric cancer tissues compared to adjacent nontumor tissues (Figure 1A, P<0.05). In addition, higher AFAP1-AS1 expression was observed in gastric cancer patients with distant metastasis than patients without distant metastasis (Figure 1B, P<0.05). Furthermore, we explored AFAP1-AS1 expression in four gastric cancer cell lines (HGC-27, SGC-7901, MKN-45, BGC-823) and normal gastric epithelial cell line GES-1 by qRT-PCR. Our data showed that AFAP1-AS1 expression was significantly increased in gastric cancer cell lines compare to GES-1 cells (Figure 1C, P<0.05). These findings suggested that AFAP1-AS1 was related to gastric cancer and it might act as an oncogene.

*AFAP1-AS1 is associated with clinical features and overall survival of gastric cancer patients*

To analyze whether AFAP1-AS1 was associated with the progression of gastric cancer, we investigated its relationship with clinical features and overall survival. The median level of AFAP1-AS1 expression was used as a cutoff value to divide all patients into two groups (high AFAP1-AS1 expression group: AFAP1-AS1 expression≥cutoff point; low AFAP1-AS1 expression group AFAP1-AS1 expression<cutoff point). We found that high AFAP1-AS1 expres-
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Table 1. Association of IncRNA AFAP1-AS1 expression with clinicopathological features in gastric cancer

<table>
<thead>
<tr>
<th>Clinicopathological features</th>
<th>No. of cases</th>
<th>AFAP1-AS1 expression</th>
<th>Chi-square value</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>&lt;60</td>
<td>46</td>
<td>25</td>
<td>0.946</td>
<td>0.331</td>
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<tr>
<td>≥60</td>
<td>41</td>
<td>18</td>
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<td></td>
</tr>
<tr>
<td>Gender</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>59</td>
<td>28</td>
<td>0.284</td>
<td>0.594</td>
</tr>
<tr>
<td>Female</td>
<td>28</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>&lt;3 cm</td>
<td>24</td>
<td>13</td>
<td>0.298</td>
<td>0.585</td>
</tr>
<tr>
<td>≥3 cm</td>
<td>63</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Well+Moderate</td>
<td>48</td>
<td>27</td>
<td>1.995</td>
<td>0.158</td>
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<tr>
<td>Poor and others</td>
<td>39</td>
<td>16</td>
<td></td>
<td></td>
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<tr>
<td>Lymph node invasion</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Absent</td>
<td>57</td>
<td>33</td>
<td>4.744</td>
<td>0.029</td>
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<tr>
<td>Present</td>
<td>30</td>
<td>10</td>
<td></td>
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<tr>
<td>Distant metastasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>60</td>
<td>35</td>
<td>6.137</td>
<td>0.013</td>
</tr>
<tr>
<td>Present</td>
<td>27</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNM stage</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>I+II</td>
<td>35</td>
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<td>8.587</td>
<td>0.003</td>
</tr>
<tr>
<td>III+IV</td>
<td>52</td>
<td>19</td>
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</table>

Expression was correlated with lymph node invasion, distant metastasis, and advanced TNM (Table 1, P<0.05). However, there are no correlation between AFAP1-AS1 expression level and other parameters, such as age, gender, tumor size and differentiation in gastric cancer (Table 1, P>0.05). Furthermore, Kaplan-Meier survival analysis and log-rank test was performed to detect the correlation between AFAP1-AS1 expression and gastric cancer patients’ overall survival. We found that gastric cancer patients with high AFAP1-AS1 expression had a shorter overall survival time than those with low AFAP1-AS1 expression (Figure 2, P<0.05).

Knockdown AFAP1-AS1 inhibits the proliferation and invasion in gastric cancer cells

To explore the critical role of AFAP1-AS1 in gastric cancer, we designed two siRNAs to knockdown the AFAP1-AS1 expression in SGC-7901 and BGC-823 cells, and the silencing efficiency was then confirmed by qRT-PCR (Figure 3A, P<0.05). As shown in Figure 3B, MTT assay showed that knockdown of AFAP1-AS1 significantly suppressed SGC-7901 and BGC-823 cells proliferation compared to cells transfected with si-NC (P<0.05). Flow cytometric analysis revealed that cell apoptosis was obviously induced after inhibition of AFAP1-AS1 in SGC-7901 and BGC-823 cells (Figure 3C, P<0.05). Moreover, transwell invasion assay suggested that cell invasion was markedly suppressed in SGC-7901 and
AFAP1-AS1 promote EMT in gastric cancer

Figure 3. Knockdown of IncRNA AFAP1-AS1 expression suppresses gastric cancer cell proliferation and invasion in vitro. A. qRT-PCR analysis of AFAP1-AS1 expression following treatment of gastric cancer cells with siRNAs targeting AFAP1-AS1. B. MTT assay showing AFAP1-AS1 knock down inhibited cell proliferation of gastric cancer cells. C. Flow cytometry was used to examine the cell apoptosis of gastric cancer cells transfected with si-AFAP1-AS1 or si-NC. D. Transwell invasion assay was performed to investigate the invasive ability of gastric cancer cells transfected with si-AFAP1-AS1 or si-NC. *P<0.05.
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E-cadherin
N-cadherin
Vimentin
GAPDH

Figure 4. Knockdown of lncRNA AFAP1-AS1 affect EMT process. Knockdown of AFAP1-AS1 increased the expression of E-cadherin, while reduced the expression of N-cadherin and Vimentin.

BGC-823 cells transfected with si-AFAP1-AS1 compared to cells transfected with si-NC (Figure 3D, P<0.05). These findings suggested that knockdown of AFAP1-AS1 could inhibit tumor proliferation and invasion in vitro.

Knockdown AFAP1-AS1 affects gastric cancer cell EMT

Epithelial-to-mesenchymal transition (EMT) is a tumor promotion process that mediates tumor invasion and metastasis [11]. In this study, we used western blot to determine EMT associated gene expression (epithelial marker E-cadherin, mesenchymal markers N-cadherin and Vimentin) in si-AFAP1-AS1 transfected SGC-7901 and BGC-823 cells. Our data showed that decreased AFAP1-AS1 expression induced E-cadherin expression and decreased N-cadherin and Vimentin expression (Figure 4, P<0.05). Those data suggested that the pro-cancer action of AFAP1-AS1 might be mediated by EMT process.

Discussion

LncRNAs are usually defined as transcribed non-coding RNAs longer than 200 nucleotides [4]. Emerging evidence reveal that IncRNAs may play a significant role in multiple physiological and pathological processes, including malignant diseases [12]. Recently, lots of new IncRNAs proved to play critical roles in tumor formation and progression. For example, Lu et al suggested that upregulated IncRNA BC03-2469 enhanced carcinogenesis and metastasis of esophageal squamous cell carcinoma through regulating hTERT expression [13]. Shao et al found that highly expressed IncRNA CRNDE promoted cell proliferation through Wnt/β-catenin signaling in renal cell carcinoma [14]. Yang et al showed that IncRNA ROR could promote the resistance of radiotherapy for human colorectal cancer cells by targeting the P53/miR-145 pathway [15]. However, to the best of our knowledge, studies of IncRNAs in gastric cancer were seldom reported.

LncRNA AFAP1-AS1 was derived from the antisense strand of DNA at the coding gene locus of AFAP1 which can regulate actin filament integrity and act as an adaptor protein linking Src family members and other signaling proteins to actin filaments [16]. Recent studies showed that AFAP1-AS1 play important roles in tumor progression. For example, Bo et al found that upregulated AFAP1-AS1 expression was associated with progression and poor prognosis of nasopharyngeal carcinoma [17]. Luo et al indicated that AFAP1-AS1 was upregulated and promoted esophageal squamous cell carcinoma cell proliferation and inhibits cell apoptosis [18]. Zhang et al showed that AFAP1-AS1 could indicate a poor prognosis of hepatocellular carcinoma and promoted cell proliferation and invasion via upregulation of the RhoA/Rac2 signaling [19]. However, little is known concerning the potential role of AFAP1-AS1 in the development and progression of gastric cancer.

In the present study, our data showed that the expression of AFAP1-AS1 was obviously increased in gastric cancer tissues and cell lines, and increased expression of AFAP1-AS1 in gastric cancer was associated with lymph node invasion, distant metastasis, advanced TNM stage, and poor prognosis, those results were consistent with previous studies. Our data revealed that AFAP1-AS1 could act as an oncogene in gastric cancer. To further explore the role of AFAP1-AS1 in gastric cancer, we inhibited AFAP1-AS1 expression in gastric cancer cell lines using siRNA. We found that decreased expression of AFAP1-AS1 obviously suppressed gastric cancer cell proliferation and invasion in vitro. Moreover, flow cytometric analysis revealed that the inhibitory effect of AFAP1-AS1 silencing on the proliferation of gastric cancer cells by inducing cell apoptosis.

EMT is a major mechanism contributing to increasing cell invasion and metastatic potential
AFAP1-AS1 promote EMT in gastric cancer

of cancer cells [11]. Recently, lots of studies showed that IncRNA play important roles in EMT progression. For example, Zheng et al showed that high expression of IncRNA PVT1 promoted invasion by inducing EMT in esophageal cancer [20]. Kong et al suggested that IncRNA H19 promoted EMT by functioning as miRNA sponges in colorectal cancer [21]. Xiao et al revealed that IncRNA UCA1 promoted EMT of breast cancer cells via enhancing Wnt/beta-catenin signaling pathway [22]. In the present study, our data showed that AFAP1-AS1 knockdown upregulated E-cadherin expression while downregulated N-cadherin and Vimentin expression. These findings demonstrated that AFAP1-AS1 affects gastric cancer cell proliferation and invasion partly through the EMT process.

In conclusion, we found that AFAP1-AS1 significantly contributes to gastric cancer progression. Inhibition of AFAP1-AS1 suppressed gastric cancer cell proliferation, invasion, and EMT. These new findings suggested that AFAP1-AS1 may be used as a potential prognostic and therapeutic target of gastric cancer.

Disclosure of conflict of interest

None.

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