AFAP1-AS1 is upregulated and promotes esophageal squamous cell carcinoma cell proliferation and inhibits cell apoptosis

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Abstract
Recent findings indicate that long noncoding RNAs (lncRNAs) were dysregulated in many kinds of tumors including esophageal squamous cell carcinoma (ESCC). LncRNA AFAP1-AS1 was found to be upregulated in hepatocellular carcinoma (HCC), lung cancer, colorectal cancer, esophageal adenocarcinoma (EAC), pancreatic ductal adenocarcinoma, and nasopharyngeal carcinoma, while its clinical value and potential function in ESCC are still unknown. Expression of AFAP1-AS1 was measured in 65 ESCC tissues and corresponding noncancerous tissues by quantitative real-time polymerase chain reaction, which revealed that AFAP1-AS1 expression was markedly elevated in ESCC tissues and significantly associated with advanced TNM stage (P = 0.004) and larger tumor size (P = 0.040). Moreover, by knocking down AFAP1-AS1 expression in ESCC cells, the proliferation and colony-forming ability were inhibited and cell apoptosis was induced. Our data indicated the first time that AFAP1-AS1, a novel oncogene, was remarkably upregulated and played a critical role in the progression of ESCC.

Introduction
Esophageal squamous cell carcinoma (ESCC) is one of the main causes of cancer death in China [1]. Owing to its increasing incidence and worse prognosis, more and more researchers are committed to the early detection of ESCC. Recently, increasing evidence has demonstrated that long noncoding RNAs (lncRNAs), comprise noncoding RNAs longer than 200 nucleotides in length, are pervasively transcribed in the genome and may participate in the regulation of cellular processes as crucial factors, such as cellular differentiation, proliferation, cell cycle regulation, and metastasis [2]. Simultaneously, many lncRNAs are demonstrated to be differentially expressed in a series of cancers, such as MALAT-1 in lung cancer [3], HULC in hepatocellular carcinoma [4], and HOTAIR in pancreatic cancer [5]. In addition, numerous evidence indicated that lncRNAs could take part in a broad range of signal pathways and act as either oncogene or tumor suppressor gene depending on their targets. However, to the best of our knowledge, studies of lncRNAs in ESCC were seldom reported.
AFAP1-AS1, which is derived from the antisense strand at the AFAP1 coding gene locus, has been reported to be upregulated in esophageal adenocarcinoma (EAC) tissues and cell lines [6]. Moreover, inhibition of its expression in EAC cells resulted in diminished cell growth, migration, invasion, along with increased apoptosis. On the basis of previous study, our current research focuses on the role of AFAP1-AS1 in ESCC. We found that the expression of AFAP1-AS1 was dramatically upregulated in ESCC tissues and cell lines. Further functional studies revealed that knockdown of AFAP1-AS1 expression in ESCC cells could result in diminished cell growth and increased apoptosis, which suggested that AFAP1-AS1 was a potential oncogene of ESCC.

**Materials and Methods**

**Patients and tissue specimens**

A total of 65 patients, who underwent radical surgery for ESCC at Huai’an First People’s Hospital, Nanjing Medical University (Huai’an, China), were selected to participate in this study. Both ESCC and corresponding adjacent specimens were collected before adjunctive therapy and the diagnosis of ESCC was confirmed by histopathology. Data of all patients including age, gender, history of smoking and drinking, ESCC tumor size, and pTNM stage were obtained from clinical material and pathology reports in 2012. After surgical resection of ESCC, the specimens were immediately collected and frozen at −80°C.

**Ethical approval of the study protocol**

This research was in accordance with the requirements of the Declaration of Helsinki. Written informed consent was received from the ESCC patients before specimen collection. Our study followed the institutional ethical guidelines approved by Huai’an First People’s Hospital, Nanjing Medical University (Huai’an, China).

**Cell culture**

Two esophageal carcinoma cell lines (ECA-109 and TE-1) were purchased from Shanghai Institutes for Biological Sciences (Shanghai, China), while a normal human esophageal epithelial cell line (HEEC) was obtained from ScienCell Research Laboratories (Carlsbad, CA 92011, USA). All the cell lines were maintained according to the vendor’s instructions. ECA-109 and TE-1 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco-BRL, USA), supplemented with 10% FBS, 100U/ml penicillin sodium, and 100 mg/mL streptomycin sulfate. All cells were cultured in a 37°C incubator containing 5% CO₂. The morphological changes of cells were observed daily under the inverted microscope. The medium were replaced every 3 days to discard the cells which were not adherent.

**Cell transfection**

After reaching more than 50% confluence, the ESCC cell lines were transfected with specific siRNA oligonucleotides. Three different siRNAs were designed to ensure transfection efficiency and avoid off-target effects. After verification, two siRNAs were thought to be appropriate for AFAP1-AS1 knockdown (Fig. 2B) (Invitrogen, Grand Island, NY, USA). Negative control siRNA (si-NC) was purchased from Invitrogen at the same time. Cells were seeded at 6-well plates for 24 h and then transfected with designed siRNA (100 nmol/L) and si-NC (100 nmol/L), respectively, by Lipofectamine RNAi MAX in serum-free medium, according to the manufacturer’s protocols (Invitrogen, Grand Island, NY, USA). Cells, after transfection, were harvested for following analyses. The sequences of the AFAP1-AS1 targeting siRNAs are summarized in Table 1.

**RNA isolation and qRT-PCR**

Total RNA was isolated from tissues or cultured cells treated with Trizol reagent (Life Technologies, Carlsbad, CA, USA). One microgram of total RNA was used for the reverse transcription reaction in a final volume of 20 μL with random primers under standard conditions using PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Dalian, China). 1 μL of the corresponding cDNA was used for subsequent qRT-PCR reactions using SYBR Premix Ex Taq (Takara, Dalian, China) according to the manufacturer’s instructions. The expression of GAPDH was used to normalize the results. The PCR amplification was performed for 40 cycles of 95°C for 5 sec, 60°C for 34 sec, and 68°C for 20 sec on an ABI 7500 Real-Time

| Primers used for qRT-PCR | Sequence |
|--------------------------|---------|
| GAPDH F                  | GGGAGGCAAAAGGGGTCTAT |
| GAPDH R                  | GAGTCCTCCACGATACCAA  |
| AFAP1-AS1 F              | AGCCTGTAATCAGCACCAC  |
| AFAP1-AS1 R              | GTTCATACCCGCTGTCC     |

Table 1. The sequence for primers and siRNA.

| siRNAs oligonucleotides | Sequence          |
|-------------------------|-------------------|
| si-AFAP1-AS1-1#         | AUUUGAUUGCCAGUUCAGUAGGCG |
| si-AFAP1-AS1-2#         | GCAUGUAGCUAUCUGACUUGCUCUGAA |
| si-AFAP1-AS1-3#         | CAACACUGCCUCCUCCUCUAAA |
PCR System (Applied Biosystems, Foster City, CA). All reactions were run in triplicate and data were analyzed using the comparative cycle threshold (CT) method. The primer sequences are summarized in Table 1.

**Cell proliferation assays**

For the cell proliferation assay, a density of 3000 cells per well was seeded in 96-well plates at day 0 (24 h after siRNA transfection). Cell proliferation was determined at 24, 48, 72, and 96 h and measured by Cell Proliferation Reagent Kit I (Roche, Basel, Switzerland). For the colony formation assay, 500 transfected cells were plated into a 6-well plate and incubated in DMEM containing 10% FBS, being replaced every 4 days. Two weeks later, colonies fixed in methanol were stained with 0.1% crystal violet (Sigma-Aldrich, St. Louis, MO) for 15 min. The colony formation was then manually counted. Three independent experiments were carried out. The formula for the colony formation ratio was as follows: Ratio = Numbers of Colony/Initiative Cells × 100%.

**Apoptosis assay**

ECA109 or TE-1 cells for cell apoptosis analysis were collected 48 h after transfection with si-AFAP1-AS1 or negative control. After staining with FITC-Annexin V and PI, the apoptosis assay was performed using the FITC-Annexin V Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer’s recommendations. Cells were then analyzed with a FACScan flow cytometry system (BD Biosciences, San Jose, CA, USA) equipped with Cell Quest software (BD Biosciences, San Jose, CA, USA). The relative ratio of early apoptotic cells and late apoptotic cells were compared to negative control transfectant, respectively.

**Statistical analysis**

The SPSS 17.0 software (IBM, Chicago, IL) was used to determine statistical difference in each experiment. The result was expressed as mean ± SD. Significance between groups was tested using paired Student’s t test, Wilcoxon test or Pearson’s chi-squared test. P < 0.05 was considered to be statistically significant.

**Results**

**AFAP1-AS1 is upregulated in ESCC tissues and correlated with tumor size and TNM stage**

The expression of AFAP1-AS1 in 65 paired ESCC tissues and corresponding adjacent tissues was observed by qRT-PCR, which showed that AFAP1-AS1 expression in ESCC was significantly elevated in 73.84% (48 of 65, fold ≥1.0) (P < 0.01) (Fig. 1A and B). Moreover, we evaluated the potential correlation between AFAP1-AS1 expression and patients’ clinical features that are shown in Table 2. Importantly, high expression of AFAP1-AS1 in ESCC was associated with tumor size (P = 0.040) and advanced TNM stage (P = 0.004). However, other parameters, such as gender (P = 0.451), age (P = 0.449), drinking status (P = 0.508), and smoking status (P = 0.880) were not associated with AFAP1-AS1 in ESCC.

**AFAP1-AS1 promotes ESCC cells proliferation in vitro**

To investigate whether AFAP1-AS1 has an effect on the proliferation of ESCC cells, we examined AFAP1-AS1 expression level in two ESCC cell lines (ECA109, TE-1) and one normal cell line (HEEC). As shown in Figure 2A, the expression levels of AFAP1-AS1 in ECA109 and

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**Figure 1.** Relative AFAP1-AS1 expression in esophageal squamous cell carcinoma (ESCC) tissues (A, B). Relative AFAP1-AS1 expression in ESCC tissues (n = 65) compared with corresponding nontumor tissues (n = 65). AFAP1-AS1 expression was examined by qPCR and normalized to GAPDH expression. Results were presented as Δ cycle threshold (CT) in tumor tissues relative to normal tissues. (B) Relative AFAP1-AS1 expression in ESCC tissues (n = 65) compared with corresponding nontumor tissues (n = 65). AFAP1-AS1 expression was classified into two groups. Positive ΔΔCT meant high AFAP1-AS1 expression. Negative ΔΔCT meant low AFAP1-AS1 expression.
TE-1 cells were higher than HEEC cells. Afterward, chemically synthesized siRNAs were used to knockdown AFAP1-AS1 expression in ECA109/TE1 cells, respectively (Fig. 2B). MTT assay was used to measure cell proliferative activity, which showed that the growth and proliferation of ESCC cells transiently transfected with siRNA1#, and siRNA2# were significantly inhibited compared with negative control groups (Fig. 2C and D). Colony formation assay also demonstrated that the clonogenic survival rate of ECA109 and TE1 cells with AFAP1-AS1 knockdown was significantly decreased (Fig. 2E and F).

**Effect of AFAP1-AS1 on ESCC cell apoptosis**

In addition, to further examine the effect of AFAP1-AS1 on cell apoptosis, flow cytometric analysis was performed. The results showed that cell apoptosis was obviously induced after interfering AFAP1-AS1 expression in ESCC cells (Fig. 2G and H).

**Discussion**

LncRNAs are usually defined as transcribed noncoding RNAs longer than 200 nucleotides [7, 8]. Emerging evidence reveal that LncRNAs may play a significant role in multiple physiological and pathological processes, including malignant diseases [9–11]. A lot of new LncRNAs are proved to play critical roles in tumor formation and progression [11].

ESCC is one of the world’s most deadly cancers [1]. In China, ESCC is the major subtype of esophageal cancer, accounting for over 90% of the cases [12]. As a tumor including multiple biological processes, ESCC begins with a variety of genetic and epigenetic changes. Earlier studies have shown that LncRNAs were critical promoters in the tumorigenesis and progression of ESCC, such as SPRY4-IT1[13], MALAT1[14], and CCAT2[15]. Wang et al. [16] recently found that the expression of LncRNA MALAT1 were higher in ESCC tissues and cell lines. Downregulation of MALAT1 decreased the expression of β-catenin, Lin28, and EZH2 genes, while overexpressed EZH2 could reverse such suppressive effect, indicating the promotion mechanism of MALAT1 in ESCC. Other researchers also discovered that several LncRNAs could serve as tumor suppressor genes in ESCC, such as UCA1[17] and LET [18]. Previous studies have demonstrated that AFAP1-AS1 was dysregulated in a variety of cancers and associated with tumor progression, including EAC [6], hepatocellular carcinoma (HCC) [19], colorectal cancer [20], pancreatic ductal adenocarcinoma [21], lung cancer [22], and nasopharyngeal carcinoma [23]. In HCC, AFAP1-AS1 was proved to be highly expressed and promoted cell proliferation and invasion via upregulation of RhoA/Rac2 signaling [24]. Zeng et al.[22] also found that AFAP1-AS1 was associated with poor prognosis and promoted cell invasion and metastasis through regulation of actin filament integrity in lung cancer. Especially, AFAP1-AS1 was found extremely hypomethylated and overexpressed in Barrett’s esophagus and esophageal adenocarcinoma, which could inhibit the biologic functions of esophageal adenocarcinoma cells [6]. There has been research indicating that AFAP1-AS1 was upregulated in ESCC and predicted chemoradioresistance and poor prognosis in patients who received definitive chemoradiotherapy [25]. However, the exact function of AFAP1-AS1 in ESCC cell biology remains unknown. Here, we evaluated the potential correlations between AFAP1-AS1 expression and patients’ clinical features. The results showed that there was a significant relationship between high AFAP1-AS1 expression and tumor size, as well as advanced TNM stage. Consistent with Wu W’s research in esophageal adenocarcinoma, our experiment found that AFAP1-AS1 could promote ESCC cells’ proliferation in vitro. Furthermore, the expression of AFAP1-AS1 was measured in the nuclear and cytosolic fractions, which indicated that AFAP1-AS1 was mostly distributed in the nucleus, prompting its function in epigenetic or transcriptional regulation, such as histone modification, chromatin remodelling, and regulating target genes or transcription factors. It was previously reported in human non-small-cell lung
Figure 2. Effects of AFAP1-AS1 knockdown on viability and apoptosis of esophageal squamous cell carcinoma (ESCC) cells in vitro. (A) Relative AFAP1-AS1 expression levels of ESCC cell lines (ECA-109, TE-1) compared with that in the normal esophageal epithelium cell line (HEEC). (B) The AFAP1-AS1 expression level was determined by qPCR when ECA-109 and TE-1 cells transfected with si-AFAP1-AS1. (C, D) MTT assays were used to determine the cell viability for si-AFAP1-AS1-transfected ECA-109 and TE-1 cells. Values represented the mean ± SD from three independent experiments. (E, F) Colony-forming assays were conducted to determine the proliferation of si-AFAP1-AS1-transfected ECA-109 and TE-1 cells. (G, H) Flow cytometry assays were performed to analyze the cell apoptosis when ESCC cells were transfected with si-AFAP1-AS1 48 h later. *P < 0.05, **P < 0.01.
cancer that AFAP1-AS1 can affect cell proliferation partly through epigenetically regulating the expression of homeobox B7 (HOXB7). Very likely, AFAP1-AS1 can modulate HOXB7 expression to regulate the proliferation of ESCC cells, which still needs further validation. There is also the possibility that AFAP1-AS1 can exert its function in ESCC through binding to PRC2, considering that PRC2-related IncRNAs are involved in multiple cancers. However, the specific molecular mechanism of AFAP1-AS1 in ESCC remains to be further studied.

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Conflict of Interest
The authors declare that they have no conflict of interests.

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