Expression and functional role of long non-coding RNA AFAP1-AS1 in ovarian cancer

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Introduction

Ovarian cancer (OC) is the third most prevalent malignancy of the female reproductive system. Every year, there are many new cases diagnosed with ovarian cancer1,2. Among them, epithelial ovarian cancer (EOC) is the fifth leading cause of cancer death in women and the most lethal gynecologic malignancy in the world3,4. Despite great progress in surgical technique, diagnostic method, and new chemotherapy regimens, treatment of ovarian cancer remains a challenge5. The majority of patients are diagnosed at advanced stage, which is the important reason of high mortality rate of ovarian cancer6. Therefore, to develop an effective ovarian cancer treatment, the elucidation of the molecular pathogenesis of ovarian cancer is required.

Long noncoding RNAs (lncRNAs) are transcribed RNA molecules longer than 200 nucleotides (nt) but have no significant protein-coding potential7,8. Accumulating data strongly support the involvement of lncRNAs in cancer. Alteration of the expression or structure of lncRNAs may promote tumor formation, progression, and metastasis9. Increasing evidence has shown altered expression level of IncRNAs in various types of human cancer and dysregulated lncRNAs may function as tumor suppressors or oncogenes. For instance, Zhao et al10 observed that overexpression of HOXAT1R could promote lung cancer cell motility and invasion. Zhang et al11 found that SNHG1 significantly promotes HCC cells proliferation, cell cycle progression, and inhibits cell apoptosis by targeting p53 expression. Cheng et al12 reported that AB073614 expression was significantly upregulated in ovarian cancer tissues and predicted poor prognosis in patients with ovarian cancer. These studies suggested that lncRNAs play critical roles in human tumor progression.

Actin filament associated protein 1 antisense RNA1 (AFAP1-AS1), was the most significantly upregulated in esophageal adenocarcinoma and lung cancer, and associated with poor prognosis13,14. However, the expression level and biological function of AFAP1-AS1 in OC is still unknown. In our study, we firstly explored the effect of AFAP1-AS1 in the progression of OC.
Patients and Methods

Patients and Tissue Samples

The present study included 130 patients with primary OC who underwent surgery at The Second Hospital of Jilin University, Changchun, China. Fresh sample was cut along the long axis into two aliquots: one was snap frozen in liquid nitrogen immediately after resection and stored at -80°C for RNA extraction, and the other was paraffin embedded for histologic evaluation of cancerous contents. No patient had received chemotherapy or radiation therapy before surgery. The clinicopathological parameters are shown in Table I. The present work was approved by the Institutional Review Board of The Second Hospital of Jilin University. Written informed consent was obtained from all the members who participated in this study.

Cell Culture

Ovarian cancer cell lines SKOV3, OV90, TOV112D and ES2 were purchased from American Type Culture Collection. Cells were maintained in DMEM/F12 (Invitrogen, Carlsbad, CA, USA) supplemented with 1% sodium pyruvate (Invitrogen, Carlsbad, CA, USA), 0.2% non-essential amino acids (Invitrogen, Carlsbad, CA, USA), and 5% FBS in a humidified atmosphere containing 5% CO2 at 37°C.

Plasmid Construction and Cell Transfection

The AFAP1-AS1 sequence was subcloned into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA). AFAP1-AS1 Ectopic expression was achieved through pcDNA3.1- AFAP1-AS1 transfection using Lipofectamine® 2000 (Invitrogen, Carlsbad, CA, USA), with an empty pcDNA3.1 vector used as a control. Plasmid vectors (pcDNA3.1-AFAP1-AS1 and pcDNA3.1) for transfection were extracted using Midiprep kits (ABI, Foster City, CA, USA), and respectively transfected into OV90 cells. For siRNA transfection, OV90 cells were seeded in a 6-well culture plate at a density of 3 × 105 cells/well and transfected with the siRNAs (si-AFAP1-AS1 and si-NC) using Lipofectamine® 2000 (Invitrogen, Life Technologies, Carlsbad, CA, USA).

RNA Isolation and qRT-PCR

Total RNA was extracted from tissue samples and cultured cells with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions. The concentration, purity, and amount of total RNA were determined by ultraviolet spectrometry (ND-1000 spectrophotometer; NanoDrop Technologies, Wilmington, DE, USA). The primers were designed as follows, for AFAP1-AS1, 5’- AATGGTGTTAGGAGGGGA-3’ and 5’-CA- CACAGGGGAATGAGAGG-3’. For ACTB (β-
actin), 5'-TCACCAACTGGGACGACATG-3' and 5'-GTCACCGAGTCCATCACGAT-3'. The RT-PCR was conducted by SYBR Premix Ex TaqTM II (Takara, Dalian, Niaoning, China) on Light-Cycler (Roche, Pudong, Shanghai, China). The relative quantitative value was expressed by the $2^{-\Delta\Delta C_{T}}$ method. Each experiment was performed in triplicates and repeated three times.

**Cell Proliferation Assay**

To assess the effects of AFAP1-AS1 in ovarian cancer cells, we first seeded cells into 96-well plates with 5 x 103 cells/well and cultured overnight. Cells were then transfected with pcDNA3.1, pcDNA3.1-AFAP1-AS1, siRNA-NC and siRNA-AFAP1-AS1. Cell proliferation was measured using the CCK-8 assay (Biyuntian, Pudong, Shanghai, China) in 24 h increments for up to 96 h. Cell proliferation was also assessed by colony formation assay. The number of stained colonies was counted.

**Flow Cytometric Analysis**

Cells were transfected with AFAP1-AS1 siRNA or its respective control and harvested at 48 h after transfection. After the propidium iodide or AnnexinV/PI double-staining, the cells were assessed for cell cycle or apoptosis by using a flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA). All assays were repeated at least 3 times.

**Statistical Analysis**

All the data were presented as the mean±SD. The significance of differences was carried out by two-paired Student’s t-test. The $\chi^2$ test was applied to the examination of the relationship between AFAP1-AS1 expression levels and clinicopathologic characteristics. GraphPad 5.0 software (San Diego, CA, USA) was used for statistical analysis. $p < 0.05$ was considered statistically significant.

**Results**

**Expression Levels of AFAP1-AS1 in OC Tissues and Cell Lines**

The expression levels of AFAP1-AS1 were first evaluated in 65 paired of OC and normal tissues by real-time RT-PCR. As showed in Figure 1A, we found that tumor tissues showed aberrant upregulation of AFAP1-AS1 compared with adjacent non-tumor tissues ($p < 0.01$). We further explored the AFAP1-AS1 expression in OC cell lines. The results showed that AFAP1-AS1 expression was significantly upregulated in OC cell

![Figure 1. AFAP1-AS1 levels were upregulated in OC cells and tissues. A, Relative expression of AFAP1-AS1 in OC tissues (n=65) compared with adjacent non-tumor normal tissues (n = 65). B, AFAP1-AS1 expression was assessed by real-time PCR using SYBR Green in OC cell lines. AFAP1-AS1 levels were normalized to β-actin levels in IOSE -80 cells. **p < 0.01, *p < 0.05.](image)
AFAP1-AS1 Upregulation Associates with Aggressive Clinicopathological Parameters of Human OC

Table I summarized the association between AFAP1-AS1 expression and clinicopathological parameters in OC. The results showed that the upregulation of AFAP1-AS1 occurred more frequently in OC patients with high FIGO stage (\(p = 0.005\)) and resistance response (\(p = 0.035\)).

The Effects of AFAP1-AS1 on Proliferation of OC

To investigate the biological role of AFAP1-AS1 in OC, CCK-8 assays were used to detect the impact of AFAP1-AS1 over-expression or knockdown on proliferation of OV90 cells. We successfully enhanced AFAP1-AS1 expression in OV90 cells by transfecting an AFAP1-AS1 expression vector (pcDNA3.1-AFAP1-AS1) and inhibited AFAP1-AS1 expression in OV90 cells by transfecting AFAP1-AS1-specific siRNAs (Figure 2A,B). CCK-8 assays showed that enhanced expression of AFAP1-AS1 significantly promoted OV90 cell proliferation (Figure 2C). CCK-8 assays showed that the depletion of AFAP1-AS1 inhibited OV90 cell proliferation in a dose-dependent manner (Figure 2D). These results indicated that forced expression of AFAP1-AS1 promoted the proliferation of OC cells.

OC Cells Apoptosis was Promoted by AFAP1-AS1 Knockdown

Then, we performed flow cytometry assay to elucidate the function of AFAP1-AS1 in the regulation of cell apoptosis in the OV90 cell line. The results showed that at 48 h post-transfection,
a significantly higher apoptotic rate was found in the OV90 cells transfected with AFAP1-AS1 siRNA compared with their controls (Figure 3).

**Discussion**

Ovarian cancer has a high mortality rate, with over 20,000 new cases diagnosed and 15,000 deaths every year in the US. Despite our understanding of ovarian cancer pathogenesis has deepened through the identification of many important oncogenes and tumor suppressors, the pathophysiological mechanisms involved in OC tumorigenesis and progression have not been wholly clarified. The identification of clinically informative and actionable biomarkers of ovarian cancer is crucial for improving prediction of prognosis and prolonging survival molecular. To the best of our knowledge, this is the first study on the clinical significance and the biological functions of AFAP1-AS1 in OC.

Recently, AFAP1-AS1 was reported to be involved in cell proliferation, angiogenesis, invasion and metastasis in various types of cancers. For example, Zhang et al. reported that the expression of AFAP1-AS1 is significantly upregulated in hepatocellular carcinoma in contrast to normal tissue. Furthermore, they showed that AFAP1-AS1 promotes hepatocellular carcinoma cell proliferation and invasion via upregulation of the RhoA/Rac2 signaling. Wang et al. indicated that AFAP1-AS1 depletion resulted in the inhibition of colorectal cancer cell proliferation and colony formation. Zeng et al. also showed that AFAP1-AS1 was associated with poor prognosis and promoted cell invasion and metastasis through regulation of actin filament integrity in lung cancer. These results revealed that AFAP1-AS1 might serve as an oncogene.

In the present study, we found that AFAP1-AS1 expression levels are remarkably increased in ovarian cancer cells and ovarian cancer tissues compared with normal controls. In clinical OC tissues, we found that high AFAP1-AS1 expression level was significantly associated with advanced FIGO stage and response. Next, we investigated the function of AFAP1-AS1 in OV90 cells. Gain-of-function and loss-of-function experiments demonstrated that AFAP1-AS1 significantly promoted OC cells proliferation and inhibited cell apoptosis. Our data confirmed that AFAP1-AS1 function as an oncogene in OC.

**Conclusions**

Our findings first demonstrated that AFAP1-AS1 is upregulated in ovarian cancer tissues and cell lines, and can promote cellular proliferation, indicating that AFAP1-AS1 can serve as a potential therapeutic target for ovarian cancer.

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**Conflict of Interest**

The Authors declare that there are no conflicts of interest.
References


