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

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Abstract. – OBJECTIVE: The present study aimed to determine the expression, roles and functional mechanisms of long non-coding RNA AFAP1-AS1 (AFAP1-AS1) in the progression of ovarian cancer.

PATIENTS AND METHODS: AFAP1-AS1 expression was assessed in ovarian cancer samples and ovarian cancer cell lines by quantitative real-time PCR. The association of AFAP1-AS1 expression with clinicopathological factors was also analyzed. Gain-/loss-of-function studies were performed to investigate the expression pattern and functional role of AFAP1-AS1 in ovarian cancer. Effects of AFAP1-AS1 on cell proliferation were studied using cell-counting kit-8 assay. The effect of AFAP1-AS1 on ovarian cancer cells apoptosis was assessed by flow cytometry assay.

RESULTS: AFAP1-AS1 was highly expressed both in ovarian cancer samples and cell lines compared with their corresponding normal counterparts. High AFAP1-AS1 expression was significantly associated with response and FIGO stage. Gain-of-function and loss-of-function experiments showed that AFAP1-AS1 promoted ovarian cancer cells proliferation. Flow cytometry assay showed that knockdown of AFAP1-AS1 promoted ovarian cancer cells apoptosis.

CONCLUSIONS: Our results suggested that AFAP1-AS1 might be considered as a novel oncogene involved in ovarian cancer progression.

Key Words:
Long noncoding RNA, AFAP1-AS1, Ovarian cancer, Proliferation, Apoptosis.

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 cancer[1,2]. Among them, epithelial ovarian cancer (EOC) is the fifth leading cause of cancer death in women and the most lethal gynecologic malignancy in the world[3,4]. Despite great progress in surgical technique, diagnostic method, and new chemotherapy regimens, treatment of ovarian cancer remains a challenge[5]. The majority of patients are diagnosed at advanced stage, which is the important reason of high mortality rate of ovarian cancer[6]. 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 potential[7,8]. Accumulating data strongly support the involvement of lncRNAs in cancer. Alteration of the expression or structure of lncRNAs may promote tumor formation, progression, and metastasis[9]. Increasing evidence has shown altered expression level of lncRNAs in various types of human cancer and dysregulated lncRNAs may functionas tumor suppressors or oncogenes. For instance, Zhao et al[10] observed that overexpression of HOTAIR could promote lung cancer cell motility and invasion. Zhang et al[11] found that SNHG1 significantly promotes HCC cells proliferation, cell cycle progression, and inhibits cell apoptosis by targeting p53 expression. Cheng et al[12] 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 prognosis[13,14]. However, the expression level and biological function of AFAP1-AS1in OC is still unknown. In our study, we firstly explored the effect of AFAP1-AS1 in the progression of OC.

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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% CO_2 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'-AATGGTGGTAGGAGGGAGGA-3' and 5'-CACACAGGGGAATGAAGAGG-3'. For ACTB (β-

Table I. Association of AFAP1-AS1 expression with clinical features.

Variables	Cases (n = 130)	AFAP1-AS1 expression		
		Low expression	High expression	*p*-value
Age (year)				0.276
≤ 60	48	21	27	
> 60	82	44	38	
Histological type				0.458
Serous	86	41	45	
Others	44	24	20	
Response				0.035
Resistance	64	26	38	
Sensitivity	66	39	27	
Differentiation				0.479
Well + moderate	56	30	26	
Poor	74	35	39	
Tumor size				0.291
< 3 cm	70	38	32	
≥ 3 cm	60	27	33	
FIGO stage				0.005
I + II	64	40	24	
III + IV	66	25	41	

actin), 5'-TCACCAACTGGGACGACATG-3' and 5'-GTCACCGGAGTCCATCACGAT-3'. The RT-PCR was conducted by SYBR Premix Ex TaqTMII (Takara, Dalian, Niaoning, China) on Light-Cycler (Roche, Pudong, Shanghai, China). The relative quantitative value was expressed by the $2^{-\Delta\Delta Ct}$ 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 × 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 χ^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

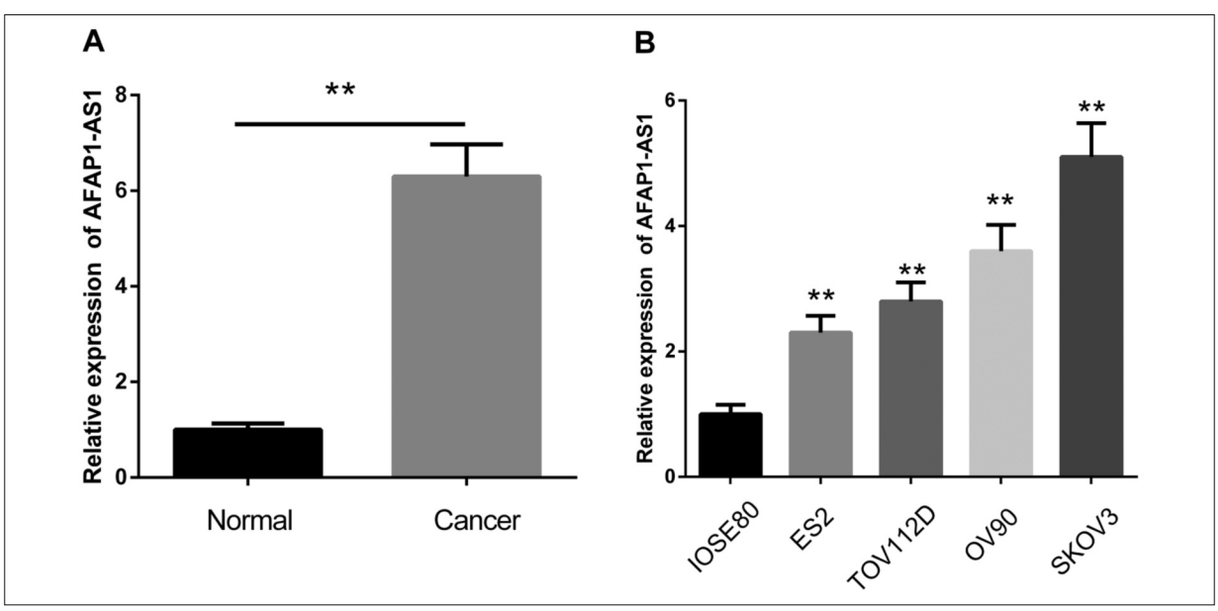

Figure 1. AFAP1-AS1 levels were upregulated in OC cells and tissues. ***A,*** Relative expression of AFAP1-AS1 in OC tissues (n=65) compared with adjacent non-tumor normal tissues (n = 65). ***B,*** AFAP1-AS1 expression was assessed by real-time PCR using SYBR Green in OC cell lines. AFAP1-AS 1levels were normalized to β-actin levels in IOSE ¬80 cells. $^{**}p < 0.01$, $^{*}p < 0.05$.

lines compared to IOSE80 cell line ($p < 0.01$, Figure 1B). Immortalized normal ovarian surface epithelial cell line IOSE80 used as a negative control.

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,

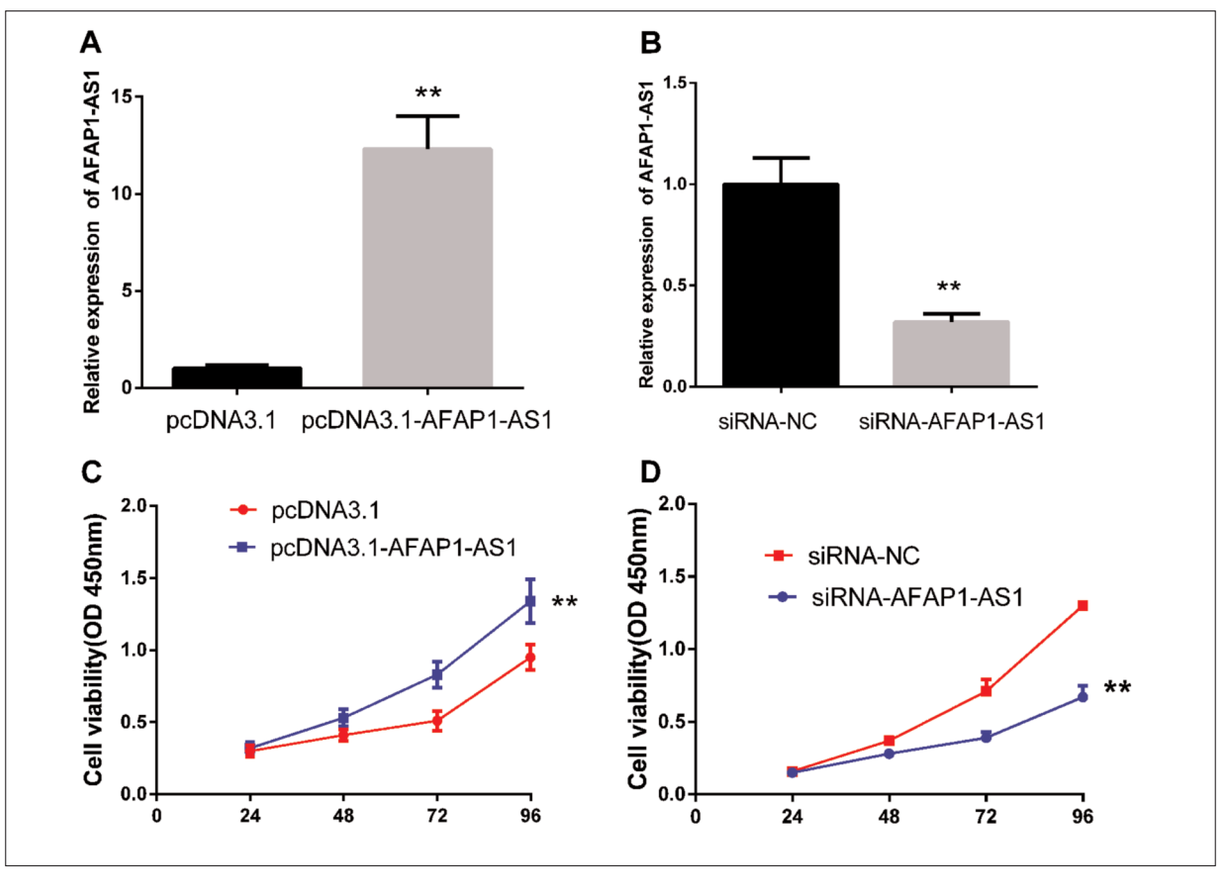

Figure 2. AFAP1-AS1 promotes OC cells proliferation. ***A,*** The relative expression of AFAP1-AS1 in OV-90 cells transfected with AFAP1-AS1 expressing vector or control vector. ***B,*** The relative expression of AFAP1-AS1 in OV-90 cells transfected with SNHG1 siRNA or negative control (NC). ***C,*** CCK-8 assays showed that AFAP1-AS1 promoted OV-90 cells proliferation. ***D,*** CCK-8 assays showed that depletion of AFAP1-AS1 inhibited OV-90 cells proliferation. $^{**}p < 0.01$, $^{*}p < 0.05$.

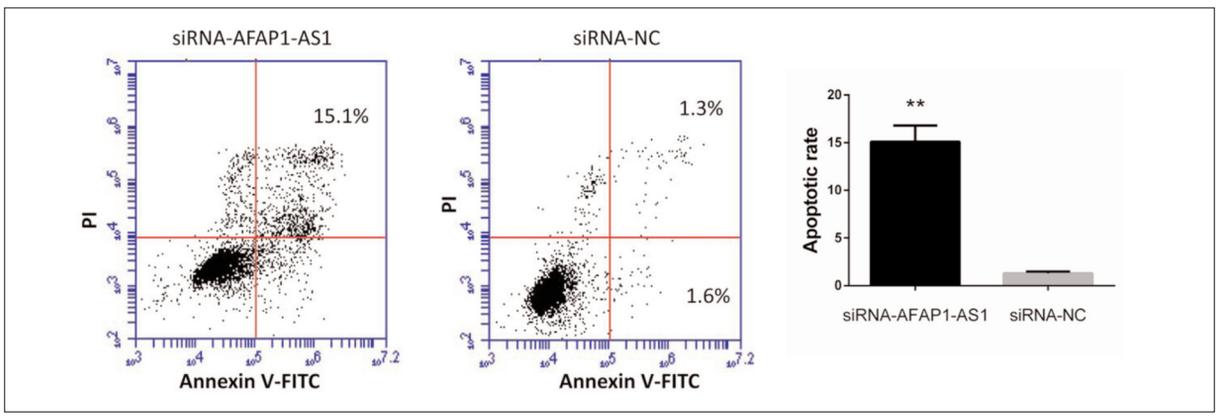

Figure 3. *A,* Flow cytometric analysis to test the role of AFAP1-AS1 on OV-90 cells apoptosis. Downregulation of AFAP1-AS1 pro¬moted OV-90 cells apoptosis. All data are showed as the mean ± SD. $^{**}p < 0.01$, $^{*}p < 0.05$.

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[15]. 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[16]. 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[17] 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[18] indicated that AFAP1-AS1 depletion resulted in the inhibition of colorectal cancer cell proliferation and colony formation. Zeng et al[19] 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.

Acknowledgements

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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