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Long noncoding RNA AFAP1-AS1 promoted osteosarcoma proliferation and invasion via upregulating BDNF

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Abstract. – OBJECTIVE: Recently, long noncoding RNAs (lncRNAs) have got much attention for their role in tumor progression. LncRNA AFAP1-AS1 was studied in this research to identify how it affects the development and proliferation of osteosarcoma.

PATIENTS AND METHODS: The mRNA expression of AFAP1-AS1 in osteosarcoma cells and tissue samples was detected by quanti[illegible]ive real-time polymerase chain reaction (qR[illegible] Moreover, cell proliferation assay and M[illegible]del assay were performed. Furthermore, the u[illegible]- lying mechanism was explored by using [illegible] PCR and Western blot assay.

RESULTS: The expression lev[illegible]AFAP1-AS[illegible] was higher in osteosarcoma [illegible]han that in adjacent tissues. In addit[illegible]the p[illegible]eration and invasion were inhibi[illegible]after [illegible]P1-AS1 was downregulated in v[illegible]Beside[illegible] and protein expressio[illegible]le[illegible]f[illegible] neurotrophic factor[illegible]NF) [illegible]duced after downregulation of [illegible]P1-AS1. F[illegible]rmore, the expression level[illegible]DNF was pos[illegible]related to the express[illegible]AP1-AS1 in o[illegible]osarcoma tissues.

CONCLU[illegible]IONS: AFAP[illegible]1 could enhance the prolif[illegible]on and invasi[illegible]osteosarcoma cells b[illegible]regulating BDNF, [illegible]ich might be a poten[illegible]therap[illegible]c target in osteosarcoma.

Key
Long [illegible]ing RN[illegible]-AP1-AS1, Osteosarcoma,

Introduction

Osteosarcoma is the most common primary [illegible]nant bone tumor with a peak incidence in ch[illegible] and adolescents, which presents with a highly malignant tendency to damage the surrou[illegible]issues[1]. Appr[illegible]ately 4 million cases [illegible]ly diagnosed of [illegible]teosarcoma per year [illegible]nd the world with a poor prognosis and a [illegible]h rate of dis[illegible]ty in youth, which brings a [illegible] burden to [illegible] patients and society[2,3]. The m[illegible]reatmen[illegible]clude surgery, chemotherapy a[illegible]apy. Despite of the developing therapeu[illegible]strategies, almost 80% of patients [illegible]ho underwent surgical treatment finally decurrent metastasis and their 5-year [illegible]rate remains 50–60%[4,5]. Therefore, it is urgent to realize the underlying molecular mechanism of osteosarcoma tumorigenesis and find out new therapeutic targets. Long noncoding RNAs (lncRNAs) are defined as ncRNAs which are more than 200 nucleotides in length. Recently, lncRNAs have got much attention for their functions in cellular processes, including the cell proliferation, cell invasion and chromatin remodeling. For example, through targeting MUC2 and up-regulating miR-34c, lncRNA AF147447 represses gastric cancer proliferation and invasion[6]. LncRNA NR_036575.1 acts as an oncogene in papillary thyroid cancer by enhancing the proliferation and migration[7]. In addition, lncRNA linc00261 functions as a tumor suppressor in gastric cancer through depressing the stability of Slug proteins and inhibiting epithelial-mesenchymal transition[8]. However, the clinical role and biological mechanism of lncRNA AFAP1-AS1 in the development of osteosarcoma remain unexplored. In this study, we found out that the expression of AFAP1-AS1 was remarkably higher in osteosarcoma tissues. In addition, AFAP1-AS1 promoted the proliferation and invasion of osteosarcoma *in vitro*. Moreover, our further investigation explored the underlying

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mechanism how AFAP1-AS1 functioned in the development and proliferation of osteosarcoma.

Patients and Methods

Patients

A total of 40 paired osteosarcoma and adjacent non-tumor tissues were sequentially gathered from patients who received surgery at Zhongnan Hospital Affiliated to Medical College of Wuhan University. Before operation, written informed consent was achieved. No radiotherapy or chemotherapy for any patients before the operation. All tissues were analyzed by an experienced pathologist. The Ethics Committee of Zhongnan Hospital Affiliated to Medical College of Wuhan University approved this study protocol.

Cell Culture

Human osteosarcoma cell lines MG-63, U2OS, Saos-2 and SOSP-9607 and osteoblastic cell line hFOB 1.19 were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Science (Shanghai, China). Culture medium consisted of 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), penicillin as well as Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Rockville, MD, USA). Culture atmosphere was an incubator containing 5% CO_2 and set at 37°C.

Cell Transfection

After synthesized virus short-hairpin RNA RNA cloned into pcDNA3.1 (GenePharma, Shanghai, China). 293T cells were used for packaging AFAP1-AS1 shRNA (shRNA) and empty vector (EV). Then shRNA (shRNA) and the empty vector (EV) were used for osteosarcoma cell transfection. Then detection of AFAP1-AS1 expression level in these cells was conducted using quantitative real-time polymerase chain reaction (qRT-PCR).

RNA Extraction and qRT-PCR

The total RNA was separated by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). And then the total RNA was reverse-transcribed to complementary deoxyribose nucleic acids (cDNA) through reverse Transcription Kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). The following are the primers using for qRT-PCR: AFAP1-AS1 primers forward 5′-AGCCTGTTGAATCAGCCAACT-3′, reverse 5′-GGTTCATACCAGCCCTGTCC-3′; β-actin primers forward 5'-CCAACCGCGAGAAGATGA-3' and reverse 5'-CCAGAGGCGTACAGGGATAG-3'. The cycle was as follows: 30 s at 95°C for 40 cycles at 95°C, 35 s at 60°C. $2^{-\Delta\Delta Ct}$ method was utilized for calculating relative expression.

Western Blot Analysis

Reagent radioimmunoprecipitation (RIPA) was utilized for extracting protein from cells. Bicinchoninic acid (BCA) protein assay kit (TaKaRa Biotech Co., Ltd., Dalian, China) was chosen for quantifying protein concentrations. The target proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Then they were incubated with antibodies after replaced to the polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Antibodies included rabbit anti-β-actin, rabbit anti-BDNF (brain derived neurotrophic factor) and goat anti-rabbit secondary antibody (Cell Signaling Technology, Danvers, MA, USA). Image J software (Bethesda, MD, USA) was performed to assess the protein expression.

Transwell Assay

5×10^4 cells in 200 μL serum-free DMEM were transformed to top chamber of an 8 μm pore size insert (Corning, Corning, NY, USA) coated with 50 μg Matrigel (BD, Bedford, MA, USA). And the lower chamber was added DMEM and FBS. After wiped by cotton swab, the top surface of chambers was immersed for 10 min with precooling methanol. Following were stain in crystal violet for 30 min. Three fields were used to count the data.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 18.0 (SPSS Inc., Chicago, IL, USA) was adopted to conduct the statistical analysis. Student *t*-text was performed to the data. Data were presented as mean ± SD (standard deviation). It was considered of statistically significance, when $p<0.05$.

Results

AFAP1-AS1 Expression Level in Osteosarcoma Tissues and Cells

First, qRT-PCR was conducted for detecting the mRNA expression of AFAP1-AS1 in 40 patients' tissues and 4 osteosarcoma cell lines. The

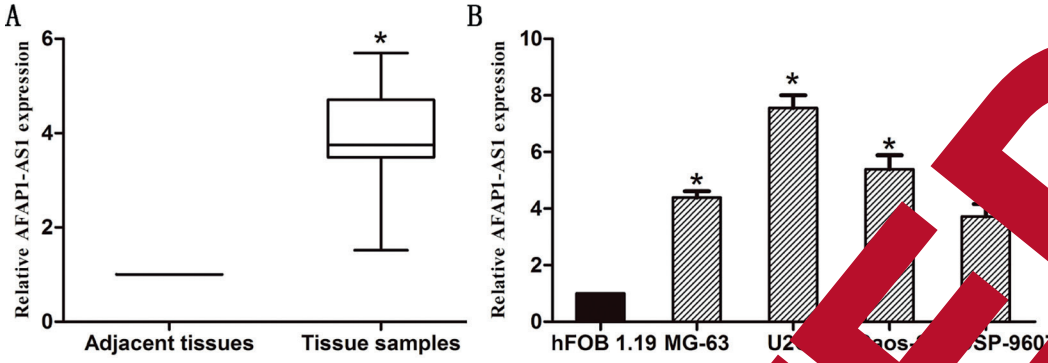


Figure 1. Expression levels of AFAP1-AS1 were increased in osteosarcoma tissues ... cell lin... AFAP1-A... xpression was significantly increased in the osteosarcoma tissues compared with adjacent t... ***B,*** Expres... AFAP1-AS1 relative to β-actin were determined in the human osteosarcoma cell lines and h... (osteoblastic c... by qRT-PCR. Data are presented as the mean ± standard error of the mean. *$p<0.05$.

results of qRT-PCR showed that AFAP1-AS1 was significantly upregulated in tumor tissue samples compared with adjacent normal tissues (Figure 1A). Meanwhile, the mRNA expression of AFAP1-AS1 in osteosarcoma cells was higher than that in hFOB 1.19 (osteoblastic cell line) (Figure 1B).

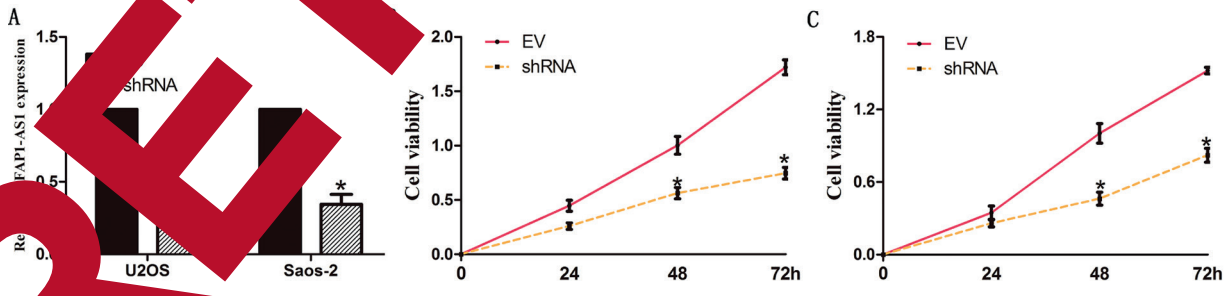
Downregulation of AFAP1-AS1 Repressed Cell Proliferation in Osteosarcoma Cells

In our study, we chose U2OS ... Saos-2 o... teosarcoma cell lines for the ... lation o... AFAP1-AS1. Then qRTPC... was ... zed for detecting the mRNA exp... ion of ... P1-AS1 (Figure 2A). Moreover, t... lts ... eration assay revealed ... t a... P1-AS1 was downregulated, th... growth ... was sig-

ni... inhibited in U2... osteosarcoma cells ...ure 2B). Meanwhile, results of cell prolifer... n assay also ... ealed that after AFAP1-AS1 ... downregula... the cell growth ability was si... antly in... ted in Saos-2 osteosarcoma cell...

Downregulation of AFAP1-AS1 ...ssed Cell Invasion in ...steosarcoma Cells

The results of Matrigel assay also revealed that after AFAP1-AS1 was downregulated in U2OS osteosarcoma cells, the number of invaded cells was remarkably decreased (Figure 3A). Meanwhile, the results of matrigel assay also showed that after AFAP1-AS1 was downregulated in Saos-2 osteosarcoma cells, the number of invaded cells was remarkably reduced (Figure 3B).



F... e 2. ...egulation of AFAP1-AS1 repressed osteosarcoma cell proliferation. ***A,*** AFAP1-AS1 expression in osteosar... a cells transduced with AFAP1-AS1 shRNA (shRNA) and the empty vector (EV) was detected by qRT-PCR. β-actin was ...s an internal control. ***B,*** Cell proliferation assay showed that downregulation of AFAP1-AS1 significantly repressed cell v... in U2OS osteosarcoma cells. ***C,*** Cell proliferation assay showed that downregulation of AFAP1-AS1 significantly re-pres... ell viability in Saos-2 osteosarcoma cells. The results represent the average of three independent experiments (mean ± standard error of the mean). *$p<0.05$.

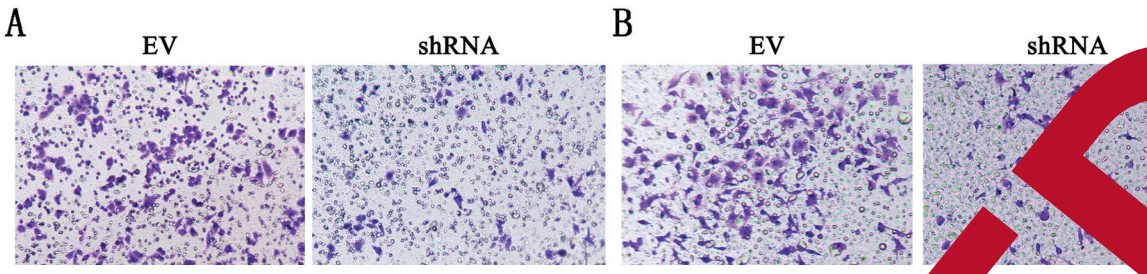


Figure 3. Downregulation of AFAP1-AS1 suppressed osteosarcoma cell invasion. ***A,*** Matri[x] [a]ssay sho[wed t]hat the nu[mber] of invaded cells was significantly decreased *via* downregulation of AFAP1-AS1 in U2OS os[teosar]coma c[ells]. ***B,*** Matrigel assay showed that the number of invaded cells was significantly decreased *via* downregulation [of] AFAP1-AS1 in Saos-[2] osteosarcoma cells. The results represent the average of three independent experiments (mean [± sta]ndard [error] of the me[an]. *$p<0.05$.

The Interaction Between BDNF and AFAP1-AS1 in Osteosarcoma

qRT-PCR results showed that compared with empty vector (EV) group, expression level of BDNF in osteosarcoma cells was lower in AFAP1-AS1 shRNA (shRNA) group (Figure 4A). Western blot assay found out that after AFAP1-AS1 [was] d[own]regulated, the protein expression level of BD[NF] [co]uld be downregulated (Fig[ure 4B]). In additi[on], [we] further found that th[e BDN]F expression in [o]steosarcoma tissues [was] obviously high[er] than that in adjacent tissues (Figure 4C). F[urth]ermore, correlation analysis demonstrated tha[t th]e BDNF expression level was

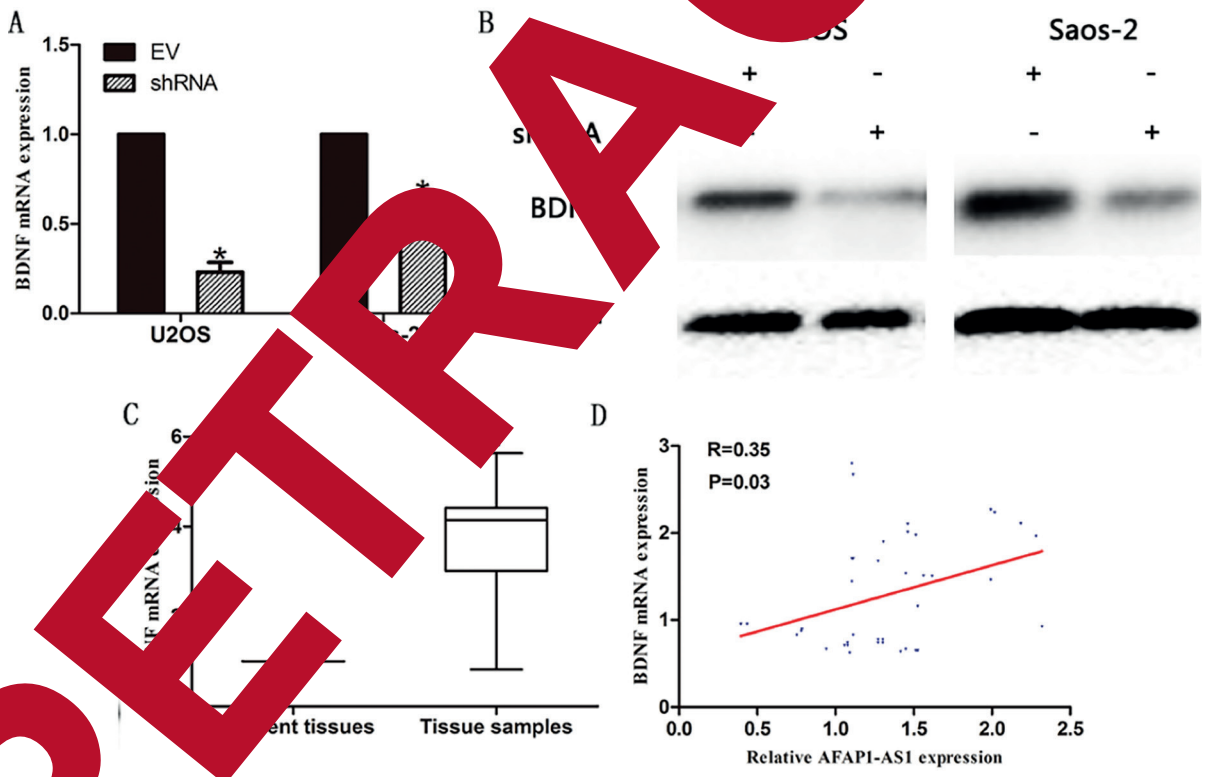


Figure 4. [Co]rrelation between AFAP1-AS1 and BDNF. ***A,*** qRT-PCR results showed that BDNF expression was lower in [AF]AP1-AS1 shRNA (shRNA) compared with the empty vector (EV). ***B,*** Western blot assay revealed that BDNF protein [expr]ession was reduced in AFAP1-AS1 shRNA (shRNA) compared with the empty vector (EV). ***C,*** BDNF was significantly [up]-regulated in osteosarcoma tissues compared with adjacent tissues. ***D,*** The expression level of BDNF was positively associated [with] AFAP1-AS1 in osteosarcoma tissues. The results represent the average of three independent experiments Data are presented as the mean ± standard error of the mean. *$p<0.05$.

positively correlated with the expression level of AFAP1-AS1 in osteosarcoma tissues (Figure 4D).

Discussion

A plenty of lncRNAs have been revealed to play an important role in the oncogenesis and progression of osteosarcoma. For example, lncRNA TUG1 functions as a ceRNA of miR-335-5p and facilitates the migration and invasion of osteosarcoma[9]. By sponging miR-326, lncRNA SNHG1 facilitates tumorigenesis in osteosarcoma through regulation of NOB1 expression[10]. LncRNA MEG3 inhibits the proliferation and metastasis of osteosarcoma through depressing Notch and TGF-beta signaling pathway[11]. Knockdown of lncRNA NEAT1 reduces the proliferation and invasion of osteosarcoma *via* inhibiting miR-194 expression[12]. LncRNA PVT1 promotes glucose metabolism, cell motility, cell proliferation and tumor progression in osteosarcoma by regulating miR-497/HK2 axis[13]. The proliferation and migration of ovarian cancer are inhibited after knockdown of lncRNA MNX1-AS1, which may offer a potential target for ovarian cancer[14]. In addition, by interacting with miR-21-5p, lncRNA [illegible]T represses the proliferation and metastasis of osteosarcoma through regulating the expression of PDCD4[15]. LncRNA AFAP-AS1 is transcript originated from the antisense strand of AFAP1 coding gene locus. Recently, several reports have revealed that AFAP1-AS1 is closely associated with various cancers. For instance, low expression of AFAP1-AS1 depresses the proliferation and induces the cell apoptosis in lung adenocarcinoma, which could offer a new therapeutic strategy for the treatment of lung adenocarcinoma[16]. Overexpression of AFAP1-AS1 induced cell apoptosis and regulated the cell proliferation in gastric cancer through PTEN/p-AKT pathway[17]. Co-expression of AFAP1-AS1 and PD-1 is associated with poor prognosis in patients with nasopharyngeal carcinoma, those patients may be ideal candidates for PD-1 immune therapy[18]. Moreover, AFAP1-AS1 promotes oncogenesis in esophageal squamous cell carcinoma by inhibiting cell apoptosis and promoting cell proliferation[19]. Our study demonstrated that AFAP1-AS1 was upregulated [illegible] in osteosarcoma tissues and cells. Furthermore, after AFAP1-AS1 was downregulated, the proliferation and invasion of osteosarcoma cells were inhibited. Above results indicated that AFAP1-AS1 could promote tumorigenesis of osteosarcoma and might act as an oncogene. Brain-derived neurotrophic factor (BDNF), as a member of the neurotrophin superfamily, has been reported to participate in the pathophysiology of nervous system. Recent reports have indicated that BDNF plays an important role in the growth and invasion of various cancers. For instance, BDNF signaling pathway plays an important role in the early recurrence of triple-negative breast cancer and affects its prognosis[20]. MicroRNA-15a-5p functioned as a tumor suppressor in human hepatocellular carcinoma by targeting BDNF[21]. BDNF was upregulated in human thyroid cancer tissues which could reverse the tumor suppressive function of miR-497[22]. Moreover, activated by STAT3, BDNF promoted the proliferation of human non-small-cell lung cancer *via* regulating TrkB signaling pathway. In the present study, BDNF expression could be inhibited after down-regulation of AFAP1-AS1. Moreover, BDNF expression in osteosarcoma tissues was positively correlated with AFAP1-AS1 expression. All those results above suggested that AFAP1-AS1 might promote the genesis of osteosarcoma *via* targeting BDNF.

Conclusions

We demonstrated that the expression of AFAP1-AS1 was remarkably upregulated in osteosarcoma tissues. In addition, AFAP1-AS1 enhanced the proliferation and invasion of osteosarcoma cells through targeting BDNF. Our findings suggested that AFAP1-AS1 could be a candidate target in the treatment of osteosarcoma.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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