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Low expression of LncRNA AFAP1-AS1 inhibits proliferation and promotes apoptosis of non-small cell lung cancer cells through inhibiting wnt signaling pathway

Ying Sun[1*], Liming Fan[1], Yixian Li[2]

[1] Department of Clinical Laboratory, Tianjin Medical University General Hospital, Tianjin 300052, China
[2] Department of Pathology, Tianjin Medical University General Hospital, Tianjin 300052, China

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ABSTRACT

To detect the effects of long non-coding ribonucleic acid (lncRNA) actin filament-associated protein 1-antisense RNA1 (AFAP1-AS1) on the proliferation and apoptosis of non-small cell lung cancer (NSCLC) A549 cells and its mechanism. 1) The expression of lncRNA AFAP1-AS1 in NSCLC A549 cells was detected *via* quantitative reverse transcription-polymerase chain reaction (qRT-PCR). 2) The changes in proliferation and apoptosis of A549 cells after low expression of lncRNA AFAP1-AS1 were detected using cell counting kit-8 (CCK-8) and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays. 3) The changes in Wnt signaling pathway proteins in A549 cells after low expression of lncRNA AFAP1-AS1 were detected using Western blotting. 1) The expression of lncRNA AFAP1-AS1 rose in A549 cells ($P<0.01$). 2) After low expression of lncRNA AFAP1-AS1, the growth of A549 cells was inhibited, and apoptosis was promoted. 3) After low expression of lncRNA AFAP1-AS1, the mRNA and protein expressions of glycogen synthase kinase (GSK) and β-catenin declined ($P<0.05$). Lowly-expressed AFAP1-AS1 inhibits the proliferation and promotes the apoptosis of NSCLC A549 cells *via* inhibiting the Wnt signaling pathway.

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Introduction

Lung cancer, causing an alarming number of deaths, is one of the most malignant cancers (1), in non-small cell lung cancer (NSCLC), is the leading cause of death of lung cancer, and accounts for approximately 80% of the total. About 75% of NSCLC patients are in the advanced stage, so the 5-year survival rate is low, which means that most NSCLC patients cannot be completely cured even after treatment (2). In addition, tumors are characterized by invasion and metastasis, which enhances their malignancy and accelerates progression, severely hindering the treatment effects, and leading to a large number of deaths (3). So far, there have been no effective methods for the diagnosis and treatment of NSCLC, especially advanced NSCLC. Therefore, it is of important significance to search for accurate prognostic markers of NSCLC, so as to improve the survival of NSCLC patients.

Long non-coding ribonucleic acids (lncRNAs) are endogenous RNAs with over 200 nucleotides in length and with no or limited protein-coding capability (4,5). Studies showed that lncRNAs play important regulatory roles in cell development. The aberrantly-expressed lncRNAs participate in cancer biology at the transcriptional and post-transcriptional levels through various mechanisms (6,7). Recently, it has been proved that lncRNAs are associated with the occurrence and development of NSCLC. For example, lncRNA PVT1 is not only overexpressed in NSCLC but also able to promote the progression of NSCLC and affect the prognosis of patients (8). A decreased expression level of lncRNA BANCR regulates the metastasis of NSCLC and is related to its prognosis (9). LncRNA FTH1P3 promotes the metastasis and invasion of NSCLC by inducing epithelial-mesenchymal transition (EMT) (10). LncRNA actin filament-associated protein 1-antisense RNA1 (AFAP1-AS1) can regulate the integrity of actin filaments, and function as an adaptor protein to link the Src family members and other signaling proteins to actin filaments (11). AFAP1-AS1 was originally discovered in esophageal adenocarcinoma in 2013 (11). It is up-regulated as an oncogene in a variety of cancers, such as hepatocellular carcinoma, esophageal cancer, colorectal cancer, gallbladder cancer and nasopharyngeal carcinoma (12-16). It is believed that AFAP1-AS1 is up-regulated in NSCLC tissues and associated with lymph node metastasis and poor prognosis in NSCLC patients (17), and it is also a potential diagnostic marker of NSCLC (18). According to mechanism research, AFAP1-AS1 promotes the migration and invasion of NSCLC by up-regulating IRF7 and RIG-I-like receptor signaling pathways (19) and induces cisplatin resistance in NSCLC through the PI3K/AKT pathway (20). In this study, the expression of AFAP1-AS1 in NSCLC cells was investigated, and whether the knockdown of AFAP1-AS1 could inhibit cell proliferation and invasion *in vitro* and tumor growth *in vivo* was explored.

The Wnt/β-catenin signal transduction pathway is an important signaling pathway in embryonic development and adult tissue homeostasis (21). According to recent studies, aberrantly expressed Wnt/β-catenin is able to drive cancer initiation and progression (22). Mutations in target genes associated with the Wnt/β-catenin signaling pathway have been found in many different types of can-

* Corresponding author. Email: stella20100616@163.com

cer (23). A recent study found that AFAP1-AS1 promotes epithelial-mesenchymal transition (EMT) and tumorigenesis in triple-negative breast cancer through the Wnt/β-catenin signaling pathway (24). However, the expression of AFAP1-AS1 in NSCLC cells and its association with the Wnt/β-catenin signaling pathway remain unclear. Therefore, whether AFAP1-AS1 participates in the proliferation and apoptosis of NSCLC cells *via* the Wnt/β-catenin signaling pathway was investigated in this study.

Materials and Methods

Cell culture

The human normal lung epithelial cell line (BEAS-2B) and human NSCLC cell line (A549) were purchased from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA) and 1% penicillin/streptomycin solution in an incubator with 5% CO_2 at 37°C.

Cell transfection

The sequences of siRNA targeting AFAP1-AS1 (shR-AFAP1-AS1) and non-target negative control (shR-NC) were provided by Life Technologies (Gaithersburg, MD, USA). After A549 cells were cultured overnight, they were transfected with 50 nM shR-AFAP1-AS1 or shR-NC using Lipofectamine 3000 transfection reagent (Life Technologies, Gaithersburg, MD, USA). Only Lipofectamine 3000 transfection reagent was added to the blank control group (Mock group).

Cell viability assay

The cell viability was determined via cell counting kit-8 (CCK-8) assay, briefly as follows. The cells were inoculated into a 96-well plate (2000 cells/well), and the optical density at 450 nm (OD_{450}) was measured at different time points using the CCK-8 kit (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's instructions.

Colony formation assay

About 1×10^4 cells were inoculated into a 6-well plate, with 3 replicates in each group. The cells were incubated in a CO_2 incubator for 2 weeks, and the medium was replaced every 3 d. Then the cells were washed twice with phosphate-buffered saline (PBS) and stained with 0.5% crystal violet. The cell colonies formed were counted under a microscope, and the colony formation efficiency was calculated: (colony count/inoculated cell count) × 100%.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay

The cells were inoculated into a 96-well plate (4×10^3 cells/well) and cultured in a humid environment with 5% CO_2 at 37°C. Then the apoptotic cells were detected using one-step TUNEL apoptosis assay kits (Beyotime, Shanghai, China), and nuclei were stained with DAPI (blue color). The fluorescence images were obtained under a fluorescence microscope (ECLIPSE Ts2R, Nikon, Tokyo, Japan). TUNEL-positive cells were quantified using ImageJ software and counted using GraphPad Prism 7.0 (La Jolla, CA, USA).

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

The cells were collected, from which the total RNA was extracted. The concentration and purity of RNA were determined using a NanoDrop-2000 spectrophotometer, and it was synthesized into complementary deoxyribose nucleic acid (cDNA) using reverse transcriptase. The transcriptional levels of proliferating cell nuclear antigen (PCNA), CyclinD1 and c-Myc were determined using SYBR Green PCR Master Mix and 7500-Fast real-time PCR system (Applied Biosystems, Foster City, CA, USA). The relative expression of mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method, with glyceraldheyde 3-phosphate dehydrogenase (GAPDH) as an internal reference. The primer sequences used are shown in Table 1.

Western blotting

After the corresponding treatment, the cells were collected, and the protein samples were extracted using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) containing protease inhibitors, followed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein samples were incubated with β-catenin (1:2,000), GSK-3β (1:1,000), p-GSK-3β (1:1,000) and β-actin (1:2,000) primary antibodies in a refrigerator at 4°C for 12-16 h, and incubated again with horse radish peroxidase (HRP)-labeled secondary antibodies (1:1,500) at room temperature for 1-2 h. Then the protein samples were washed with PBS with Tween-20, and the protein bands were detected using electrochemiluminescence (ECL).

Statistical analysis

The experimental data were expressed as mean ± standard deviation. GraphPad Prism 7.0 (La Jolla, CA, USA) was used for statistical analysis. Differences between the two groups were analyzed by using the Student's t-test. Comparison between multiple groups was done using a

Table 1. Primer sequences of qRT-PCR.

Index	Forward (5'-3')	Reverse (5'-3')
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC
PNCA	CCTGCTGGGATATTAGCTCCA	CAGCGGTAGGTGTCGAAGC
CyclinD1	GCTGCGAAGTGGAAACCATC	CCTCCTTCTGCACACATTTGAA
c-Myc	CGTCTCCACACATCAGCACAA	TCTTGGCAGCAGGATAGTCCTT
β-catenin	AAAGCGGCTGTTAGTCACTGG	CGAGTCATTGCATACTGTCCAT
GSK-3β	GGCAGCATGAAAGTTAGCAGA	GGCGACCAGTTCTCCTGAATC
AFAP1-AS1	AATGGTGGTAGGAGGGAGGA	CACACAGGGGAATGAAGAGG

One-way ANOVA test followed by Post Hoc Test (Least Significant Difference). P<0.05 suggested the statistically significant difference.

Results

Expression of AFAP1-AS1 rose in A549 cells

The expression of lncRNA AFAP1-AS1 in A549 cells was analyzed via qRT-PCR. As shown in Figure 1, the expression of lncRNA AFAP1-AS1 significantly rose in A549 cells (P<0.01).

Knockdown of AFAP1-AS1 inhibited the proliferation of A549 cells

To explore the effect of AFAP1-AS1 on the proliferation of NSCLC cells, the expression of AFAP1-AS1 was knocked down in A549 cells. It was found that the expression of AFAP1-AS1 in A549 cells could be effectively knocked down by shRNA (P<0.01, Figure 2A). Besides, the changes in A549 cell phenotype induced by AFAP1-AS1 knockdown were evaluated. The results revealed that the growth of A549 cells was obviously inhibited after AFAP1-AS1 knockdown (P<0.05, Figure 2B). In addition, the number of colonies formed declined after AFAP1-AS1 knockdown (P<0.01, Figure 2C).

Knockdown of AFAP1-AS1 markedly promoted apoptosis of A549 cells

To detect the effect of AFAP1-AS1 on the apoptosis of NSCLC cells, the expression of AFAP1-AS1 was knocked down in A549 cells, and the changes in apoptosis were evaluated. As shown in Figure 3, AFAP1-AS1 knockdown markedly promoted the apoptosis of A549 cells (P<0.05).

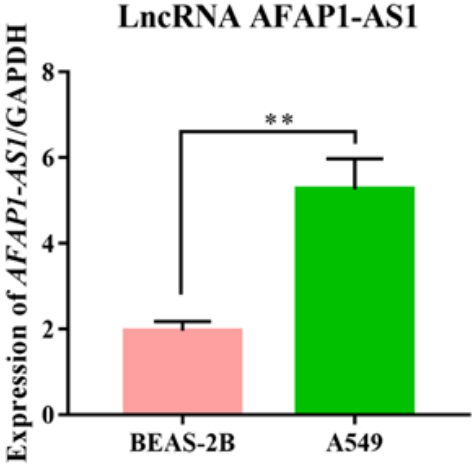

Figure 1. The results of qRT-PCR showed that the expression of lncRNA AFAP1-AS1 rose in A549 cells. Note: **P<0.01 vs. BEAS-2B cells.

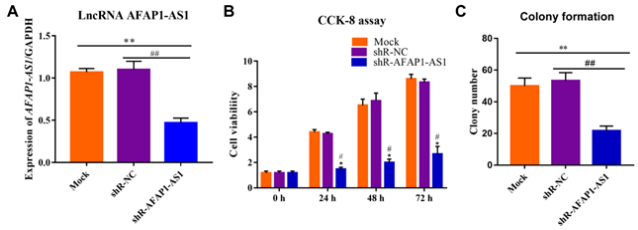

Figure 2. Knockdown of AFAP1-AS1 obviously inhibited proliferation of A549 cells. (A) Knockdown efficiency of AFAP1-AS1 with shRNA. (B) Proliferation of A549 cells after AFAP1-AS1 knockdown detected using CCK-8 assay. (C) Colony formation assay. (magnification: 40×) Note: *P<0.05, **P<0.01 vs. Mock group, #P<0.05, ##P<0.01 vs. shR-NC group.

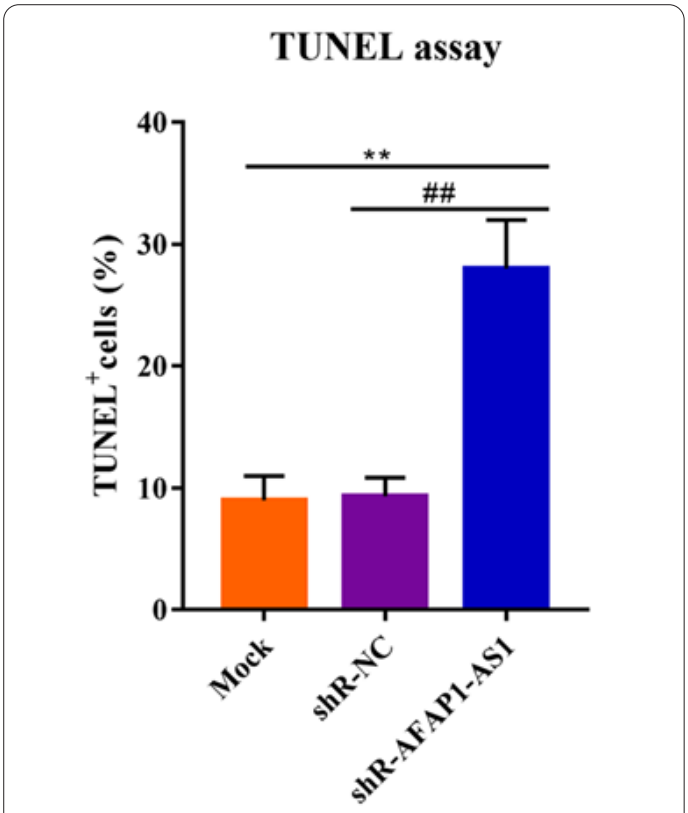

Figure 3. AFAP1-AS1 knockdown markedly promoted the apoptosis of A549 cells. (magnification: 200×) Note: **P<0.01 vs. Mock group, ##P<0.01 vs. shR-NC group.

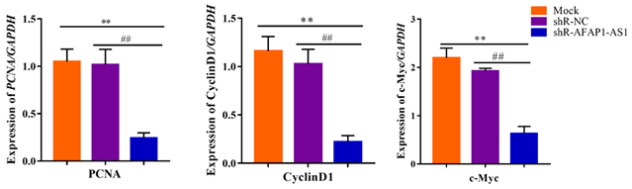

Figure 4. Effects of AFAP1-AS1 knockdown on mRNA levels of PNCA, CyclinD1 and c-Myc in A549 cells. Note: **P<0.05 vs. Mock group, ##P<0.05 vs. shR-NC group.

Effects of AFAP1-AS1 knockdown on mRNA levels of PNCA, CyclinD1 and c-Myc

To detect the effects of AFAP1-AS1 knockdown on the expressions of proliferation- and apoptosis-related genes, the mRNA levels of PNCA, CyclinD1 and c-Myc in A549 cells were determined. After the knockdown of AFAP1-AS1, the mRNA expressions of PNCA, CyclinD1 and c-Myc declined in A549 cells (P<0.01) (Figure 4).

Knockdown of AFAP1-AS1 remarkably reduced activity of the Wnt signaling pathway

After the knockdown of AFAP1-AS1 in A549 cells, the total RNA was extracted in each group, and the mRNA and protein levels of β-catenin and GSK-3β were determined via qRT-PCR and Western blotting, respectively. As shown in Figure 5, the mRNA and protein expressions of β-catenin and GSK-3β were remarkably suppressed in A549 cells after the knockdown of AFAP1-AS1 (P<0.01,

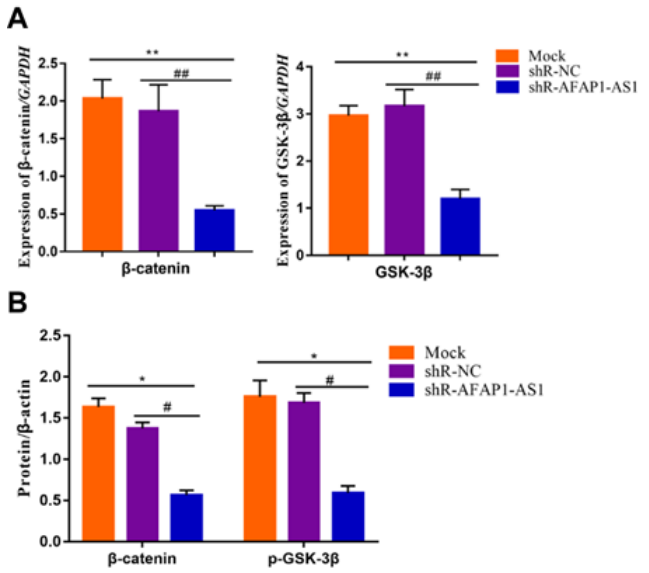

Figure 5. Knockdown of AFAP1-AS1 remarkably reduced the activity of the Wnt signaling pathway. (A) MRNA expressions of β-catenin and GSK-3β were detected using qRT-PCR. (B) Protein expressions of β-catenin and GSK-3β were detected using Western blotting. Note: **P<0.05 vs. Mock group, $^{\#\#}$P<0.05 vs. shR-NC group.

P<0.05).

Discussion

NSCLC, with a 5-year overall survival rate of no more than 15%, accounts for about 85% of all lung cancers (25). Traditional treatment methods for NSCLC include surgery, chemotherapy and radiotherapy. However, none of them are effective for advanced NSCLC. Hence, it is of great importance to identify more valuable factors for improving the clinical effects of NSCLC. According to existing reports, many biomarkers have been determined for the diagnosis or prognosis of NSCLC, among which lncRNAs re new focuses. Lots of research found that lncRNAs, as novel prognostic markers of tumors, are of great significance in the diagnosis and treatment of tumors (26). So far, more than 3,000 lncRNAs have been identified, but the function of only 1% of them has been clarified (27). Therefore, lncRNAs, as a class of molecular markers of diseases, need to be further studied.

LncRNAs are known to regulate many biological processes, such as gene expression, cell development, differentiation and apoptosis (28,29). Since AFAP1-AS1 is overexpressed in NSCLC tissues, it was speculated that AFAP1-AS1 may be related to the progression and development of NSCLC. The Wnt/β-catenin pathway plays an important role in the proliferation, survival and differentiation of cancer cells (30,31). When Wnt ligands bind to transmembrane receptors, the Wnt signal transduction can be initiated. As β-catenin accumulates in the nucleus, β-catenin/TCF/LEF transcription complexes are formed, ultimately activating Wnt target genes and initiating a series of proliferation- and apoptosis-related events (30). There is increasing evidence that many important oncogenes or tumor suppressor genes regulate the growth, migration and invasion of tumor cells by regulating the Wnt/β-catenin signaling pathway (32). In addition, lncRNAs also participate in these regulatory processes. For example, lncRNA PTCSC3 inhibits the proliferation and invasion of glioma cells by inhibiting the Wnt/β-catenin signaling pathway (33). LncRNA MALAT1 induces EMT and inhibits apoptosis of tongue cancer cells through the Wnt/β-catenin signaling pathway (34). Moreover, lncRNA MSC-AS1 regulates cell proliferation and migration in renal clear cell carcinoma by activating the Wnt/β-catenin signaling pathway ((35, 36). According to previous studies, the proliferation, invasion and metastasis of NSCLC can be enhanced through the Wnt/β-catenin signaling pathway (37). In this study, it was found that AFAP1-AS1 could promote the occurrence of NSCLC through the Wnt/β-catenin signaling pathway. The expressions of several molecules, including β-catenin, in the Wnt/β-catenin signaling pathway were detected in this study. It was found that the knockdown of AFAP1-AS1 could significantly down-regulate the expression of β-catenin in NSCLC cells, which indicates that the overexpression of AFAP1-AS1 in NSCLC can up-regulate the expression of β-catenin, thus promoting activation of the Wnt/β-catenin signaling pathway. Additionally, the expressions of proliferation- and apoptosis-related genes were determined by qRT-PCR. The results showed that the target genes associated with the Wnt/β-catenin signaling pathway, including PCNA, CyclinD1 and c-Myc. played a role in the proliferation and apoptosis of NSCLC cells. However, the function of AFAP1-AS1 in the development of NSCLC needs to be further investigated.

In summary, this study provides the first evidence that AFAP1-AS1 is highly expressed in NSCLC cell lines. In addition, the knockdown of AFAP1-AS1 by shRNA can significantly inhibit the proliferation and clone formation of A549 cells and promote their apoptosis. Silencing AFAP1-AS1 partially inhibits tumor growth *in vivo*, which serves as an oncogene in NSCLC by inhibiting the Wnt/β-catenin signaling pathway and suppressing the expression of the proliferation-related target genes.

Conclusions

In conclusion, AFAP1-AS1 is a promising potential target for clinical treatment of NSCLC. However, the clinical data were limited in this research, so a large number of clinical samples may be required for further experiments in the future.

Conflict of Interest

The authors declared no conflict of interest.

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