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Original Article

Knockdown of long non-coding RNA *AFAP1-AS1* inhibits growth and promotes apoptosis in pituitary adenomas

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Abstract: Recent studies have reported that the long non-coding RNA (lncRNA) AFAP antisense RNA 1 (*AFAP1-AS1*) is involved in various biological processes and plays a key role in regulating cancer growth and metastasis in humans. However, its effects on tumorigenesis in pituitary adenomas remain unclear. The present study investigated the expression and biological role of *AFAP1-AS1* in pituitary adenomas. We observed that the expression of *AFAP1-AS1* was considerably higher in the pituitary adenoma tissues as compared to its expression in the adjacent tissues. Additionally, knockdown of *AFAP1-AS1* inhibited the proliferation, arrested the cell cycle in the G1-to-S transition phase, and promoted apoptosis in GH3 and MMQ cells. Finally, knockdown of *AFAP1-AS1* also promoted the expression of PTEN and inhibited the expression of PI3K and p-AKT. Our results provided novel insights into the function and mechanism of action of *AFAP1-AS1* in the pathogenesis of pituitary adenomas.

Keywords: Long non-coding RNA, *AFAP1-AS1*, pituitary adenoma, PTEN/PI3K/AKT

Introduction

Pituitary adenomas are the third most commonly occurring type of intracranial tumors, whose incidence is second only to that of gliomas and meningiomas, account for 10-15% of all intracranial tumors. however, their incidence has increased in the recent years [1, 2]. Although pituitary adenomas have been histologically classified as benign tumors, some of them are invasive and are characterized by their ability to penetrate into the healthy adjacent structures such as the skull, cavernous sinus, dura mater, sphenoid sinus, third ventricle, and other locations. The mainstay of treatment for patients with pituitary adenomas is surgical removal, chemotherapy, and radiotherapy, all of which have limited effectiveness, and the reason is largely caused by pituitary adenoma metastasis [3, 4]. Additionally, the existing therapeutic methods can adversely affect the normal secretion of pituitary and even reduce pituitary function, thereby increasing the suffering of patients undergoing long-term hormone replacement therapy [5]. Thus, understanding the molecular pathogenesis of pituitary adenoma is significantly important. Substantial evidence has indicated that microRNAs and proteins act as tumor suppressor genes or oncogenes in pituitary adenomas [6, 7]; however, the role of long non-coding RNAs (lncRNAs) remains unclear.

LncRNAs participate in the regulation of crucial biological processes, such as cellular differentiation, proliferation, cell cycle, tumorigenesis, and metastasis. A previous study has shown that the expression of lncRNA taurine upregulated gene 1 (*TUG1*) was significantly upregulated in the pancreatic cancer tissues as compared to its expression in the adjacent tissues and it also activated the epithelial-mesenchymal transition (EMT) process, thereby promoting proliferation and migration of pancreatic cancer cells [8]. In osteosarcoma, the expression of lncRNA homeobox A11 antisense (*HOXA11-AS*) was upregulated. Furthermore, *HOXA11-AS* downregulation inhibited the prolif-

eration and invasion of osteosarcoma cells, and arrested the cell cycle in the G0-to-G1 transition phase in these cells [9]. The expression of MEG3 considerably decreased, whereas that of HOTAIR considerably increased in the invasive non-functioning pituitary adenomas (NFPAs) compared to their expressions in the non-invasive NFPAs [10]. Previous study indicated that *AFAP1-AS1* functions as an oncogene in cholangiocarcinoma [11] and esophageal squamous cell carcinoma [12]; however, its function and underlying mechanisms associated with tumorigenesis in pituitary adenomas remain poorly understood.

We hypothesized that the abnormity of *AFAP1-AS1* might be responsible for tumorigenesis in pituitary adenomas. In this study, we observed considerably higher levels of *AFAP1-AS1* in the pituitary adenoma tissues as compared to its levels in the adjacent tissues. Additionally, knockdown of *AFAP1-AS1* inhibited proliferation of GH3 and MMQ cells, arrested the cell cycle in the G1-to-S transition phase, and promoted apoptosis in these cells. Finally, knockdown of *AFAP1-AS1* was upregulated the expression of PTEN and downregulated the expressions of PIEK and p-AKT. Our data revealed that *AFAP1-AS1* knockdown promoted cell apoptosis, inhibited cell proliferation, and regulated the PTEN/PI3K/AKT signaling pathway in pituitary adenomas.

Materials and methods

Patients and tissue samples

Sixty patients with pituitary adenomas were recruited from Third Affiliated Hospital, Sun Yat-sen University between January 2015 and January 2017. Diagnosis of pituitary adenoma was confirmed in these patients by two pathologists independently. All experiments were approved by the Ethics Committee of Third Affiliated Hospital, Sun Yat-sen University. The pituitary adenoma tissues and adjacent normal tissues were obtained by performing surgical operation, and were immediately stored at -80°C until further use. All participants in this study signed an informed consent form.

RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Briefly, total RNA was extracted from the pituitary adenoma tissues and adjacent normal tissues using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Next, mRNA was reverse transcribed to cDNA using the PrimeScript RT Reagent Kit (Takara Bio Inc., Dalian, China), according to the manufacturer's instructions. qRT-PCR was performed to quantitate the expression of lncRNA *AFAP1-AS1* using the SYBR Premix Ex Taq (Takara Bio Inc.) and 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The expression of *AFAP1-AS1* was normalized with respect to the expression of GAPDH. Relative expression of lncRNA *AFAP1-AS1* was expressed in terms of fold-change calculated using the $2^{-\Delta\Delta Ct}$ method. Nucleotide sequences of the primers used are as follows: for lncRNA *AFAP1-AS1* (forward, 5'-AGCCTGTTGAATCAGCCAACT-3'; reverse, 5'-GGTTCATACCAGCCCTGTCC-3') and for *GAPDH* (forward, 5'-CCCATCACCATCTTCCAGG-AG-3'; reverse, 5'-GTTGTCATGGATGACCTTGG-C-3').

Cell culture and siRNA transfection

GH3 and MMQ, rat pituitary adenoma cell line, was cultured in Ham's F-12 medium (Gibco BRL, Grand Island, NY, USA) supplemented with fetal bovine serum (2.5%), horse serum (15%), glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37°C in a humid atmosphere of 5% CO_2. si-*AFAP1-AS1*-1 (5'-AUUUGAUGCCAGUUCAGUAGAGCCG-3'), si-*AFAP1-AS1*-2 (5'-CAACACCUGCCUUCCCUCCUC-UAAA-3'), si-*AFAP1-AS1*-2 (5'-GCCAUGUCAUC-UGACUGGCUCUGAA-3'), and negative control siRNA (si-NC, 5'-UUCUCCGAACGUGUCACG-UTT-3') were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). The GH3 and MMQ cells were transfected with the siRNAs (25, 50, and 100 μmol/L) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. After 48 h, *AFAP1-AS1* expression was examined using qRT-PCR to analyze the efficiency of transfection.

Cell proliferation assay

In order to analyze cell proliferation, the CCK-8 assay was performed according to the manufacturer's protocol. Briefly, 5,000 cells/well were plated in triplicate in the 96-well plates and cultured for 0, 24, 48, and 72 h post-transfection. Subsequently, the CCK-8 solution (10 μL) was added to each well. Cells were incubat-

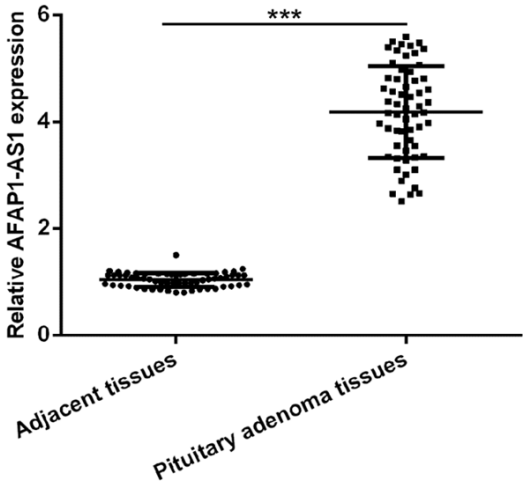

Figure 1. *AFAP1-AS1* expression in 60 paired samples of pituitary adenoma tissues and adjacent tissues analyzed using qRT-PCR. ***, $P < 0.001$ vs. adjacent tissues.

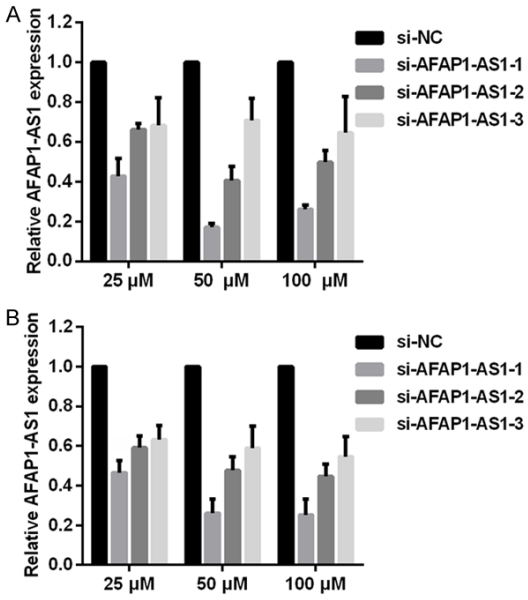

Figure 2. qRT-PCR analysis showing the expression of *AFAP1-AS1* upon transfection with different concentrations (25, 50, and 100 μmol/L) of si-*AFAP1-AS1-1*, si-*AFAP1-AS1-2*, and si-*AFAP1-AS1-3* in GH3 (A) and MMQ (B) cells.

ed at 37°C for 4 h and the absorbance at 450 nm was measured using a microplate reader.

Apoptosis and cell cycle assay

At 48-h post-transfection, measurement of apoptosis and the distribution of cells in different phases of the cell cycle were performed using flow cytometry. For measuring apoptosis, 5×10^3 cells were resuspended in the binding buffer (500 μL), and Annexin V-FITC (5 μL) and PI (5 μL) were added. The samples were mixed at 25°C. Next, the cells were incubated for 15 min in dark. For performing the cell cycle assay, the GH3 and MMQ cells were fixed in pre-cooled ethanol (70%) and incubated with ribonuclease A (200 μg/mL) for 30 min at 37°C. Next, propidium iodide (PI, 100 μL) was added and the samples were incubated in dark for 30 min at 4°C. The distribution of cells among different phases of the cell cycle and their apoptotic rate was analyzed using flow cytometry (BD Biosciences, San Diego, CA, USA).

Hoechst 33258 staining assay

To determine the effect of AFAP1-AS1 knockdown on apoptosis in the GH3 and MMQ cells, Hoechst 33258 staining assay was performed as described previously [13]. Briefly, at 48-h post-transfection, the GH3 and MMQ cells were stained by Hoechst 33258 (5 mg/mL) for 20 min at room temperature and were observed under the Leica DM IL LED fluorescence microscope (Leica, Wetzlar, Germany).

Western blotting

The expression levels of PTEN, PI3K, p-AKT, and AKT were detected using western blotting. Total protein was extracted from the GH3 and MMQ cells using ice-cold RIPA extraction and lysis buffer (Takara Bio Inc.) containing the proteinase inhibitor cocktail (Takara Bio Inc.). Next, the total protein concentration was estimated using the BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). The extracted proteins were resolved using 10% SDS-PAGE. The resolved proteins were transferred onto nitrocellulose membranes which were incubated with the respective primary antibodies (Abcam, Cambridge, MA, USA), including the rabbit anti-PTEN antibody (1/10000), rabbit anti-PI3K antibody (1/1000), rabbit anti-p-AKT antibody (1/900), rabbit anti-AKT antibody (1/5000), and rabbit anti-GAPDH antibody (1/10000). Next, the nitrocellulose membranes were incubated with the HRP-conjugated goat anti-rabbit IgG H&L secondary antibody (1/10000; Southern Biotech, Birmingham, AL, USA) for 40 min. The protein bands were visualized using ECL (Thermo Scientific). The expression of all proteins was normalized against the expression of GAPDH.

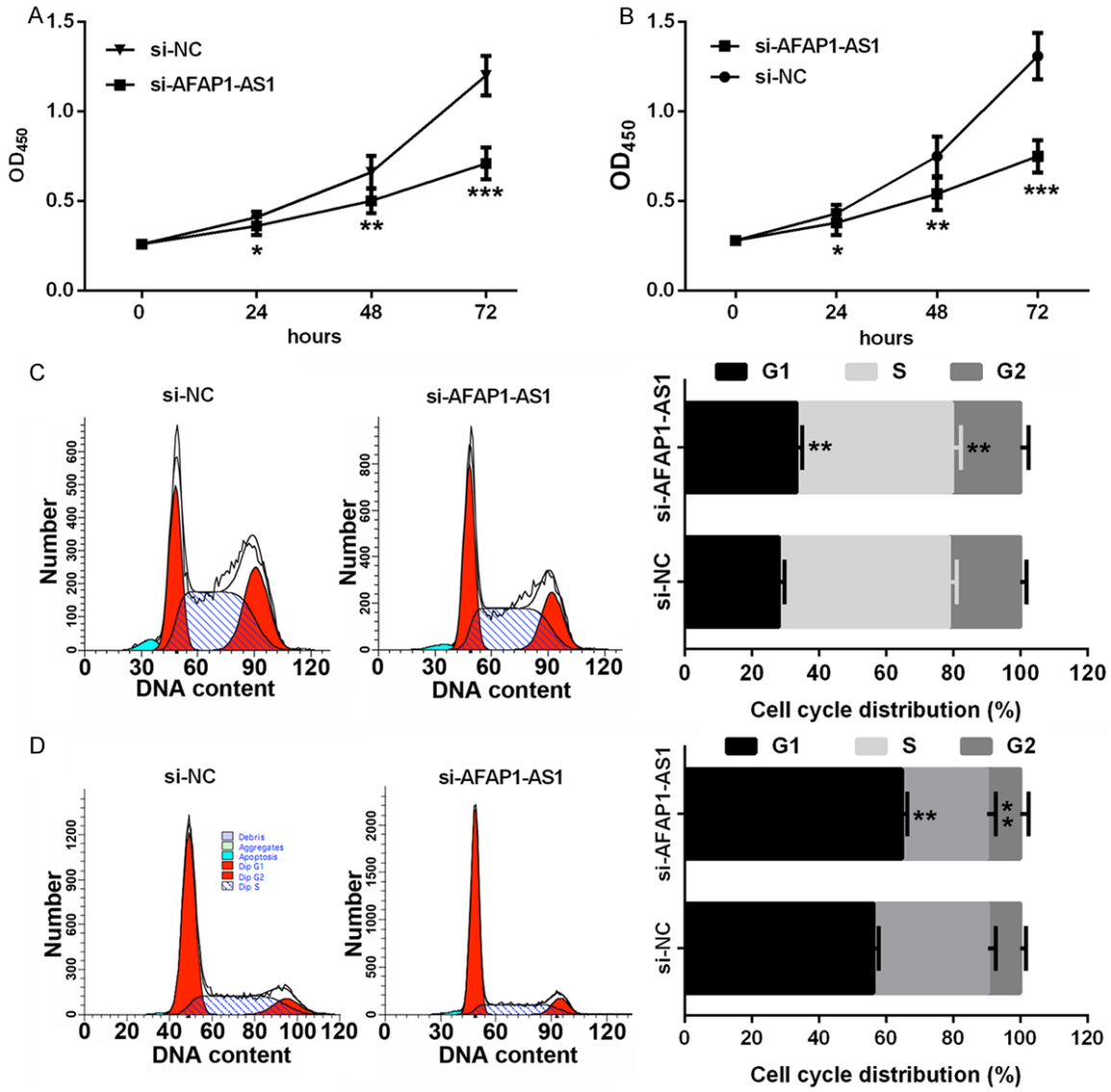

Figure 3. The effect of *AFAP1-AS1* knockdown on cell proliferation and cell cycle progression. A and B: Cell proliferation was measured 24, 48, and 72 h post-transfection using the CCK-8 assay in GH3 (A) and MMQ (B) cells. C and D: At 48-h post-transfection, the distribution of cells in different phases of the cell cycle was analyzed using flow cytometry in GH3 (C) and MMQ (D) cells. Data represent the mean ± SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ vs. si-NC group.

A *P*-value (*P*) < 0.05 was considered to be statistically significant.

Results

AFAP1-AS1 expression is upregulated in pituitary adenomas

The expression of *AFAP1-AS1* in the 60 paired samples of pituitary adenoma tissues and the adjacent tissues was analyzed using qRT-PCR (**Figure 1**). The results showed that the expression of *AFAP1-AS1* in the pituitary adenoma tissues and adjacent tissues was 1.03 ± 0.133 and 4.18 ± 0.862, respectively. This showed that the expression of *AFAP1-AS1* in the pituitary adenoma tissues was significantly higher than its expression in the adjacent tissues.

SiRNA transfection inhibits the expression of AFAP1-AS1

To investigate whether *AFAP1-AS1* had any effect on the biological functioning of the pituitary adenoma cells, three siRNAs were used to knockdown the expression of AFAP1-AS1 in the GH3 and MMQ cells (**Figure 2**). The expression of AFAP1-AS1 was significantly inhibited (*P* <

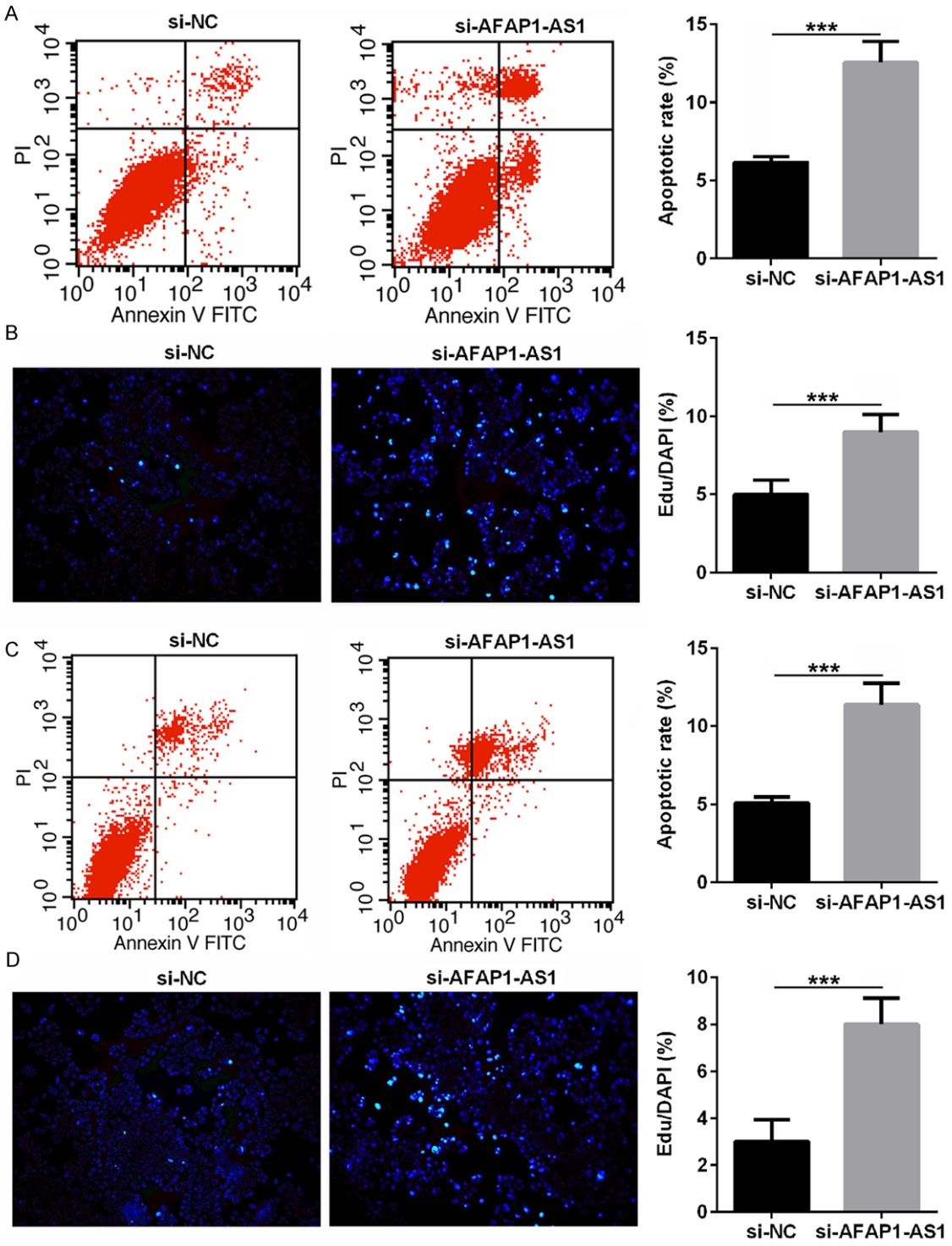

Figure 4. *AFAP1-AS1* silencing promoted apoptosis in GH3 and MMQ cells. A and B: At 48-h post-transfection, the effect of *AFAP1-AS1* knockdown on the apoptotic rate of GH3 cells was measured using flow cytometry and Hoechst assay. C and D: At 48-h post-transfection, the effect of *AFAP1-AS1* knockdown on the apoptotic rate of MMQ cells was measured using flow cytometry and Hoechst assay. ***, $P < 0.001$ vs. si-NC group.

0.05) upon transfection by si-*AFAP1-AS1-1*, si-*AFAP1-AS1-2*, and si-*AFAP1-AS1-3*, compared to its expression upon transfection with si-NC. The result also indicated that transfection with

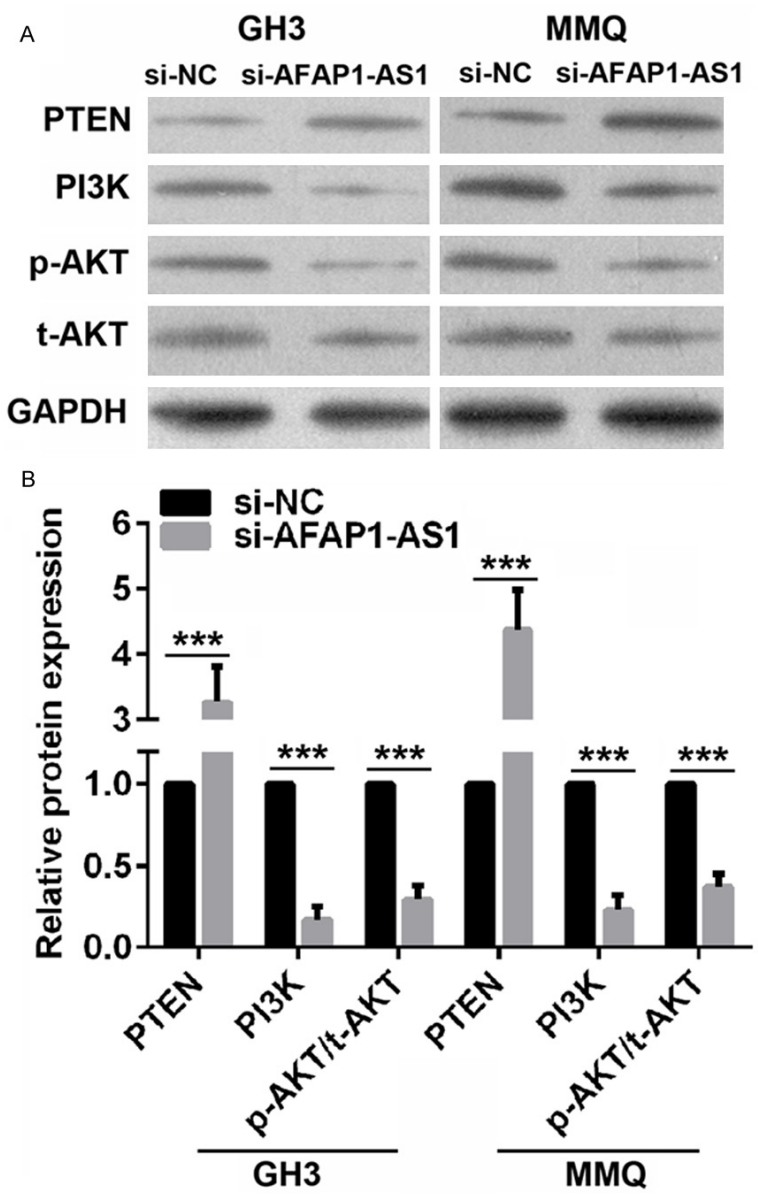

Figure 5. Knockdown of *AFAP1-AS1* regulated PTEN/PI3K/AKT signaling pathway. A: The expression of PTEN, PI3K, AKT, and p-AKT were detected by western blotting. B: The expression of PTEN, PI3K, AKT, and p-AKT were represented the mean ± SD. ***, P < 0.001 vs. si-NC group.

50 μmol/L of si-*AFAP1-AS1-2* resulted in the most effective inhibition. Thus, this concentration was used for the subsequent experiments.

Knockdown of AFAP1-AS1 inhibits cell proliferation, arrests the cell cycle in pituitary adenoma cells

The proliferation of GH3 and MMQ cells after transfection was examined by using the CCK-8 assay. The results indicated that the proliferation of GH3 and MMQ cells in the si-AFAP1-AS1 group was significantly inhibited as compared to their proliferation in the si-NC group, 24, 48, and 72 h post-transfection (P < 0.05, **Figure 3A** and **3B**). At 48 h post-transfection, the distribution of cells in different phases of the cell cycle was examined using flow cytometry. The results revealed a smaller proportion of the total cell population in the S phase and a larger proportion in the G1 phase in the si-AFAP1-AS1 group as compared to the si-NC group (P < 0.05, **Figure 3C** and **3D**).

Knockdown of AFAP1-AS1 promotes cell apoptosis in pituitary adenoma cells

At 48-h post-transfection, apoptotic rate was analyzed using Hoechst assay and flow cytometry. The results showed that the apoptotic rate of GH3 and MMQ cells in the si-*AFAP1-AS1* group was higher than that in the si-NC group (*P* < 0.05, **Figure 4**).

Knockdown of AFAP1-AS1 regulated PTEN/PI3K/AKT signaling pathway

The expressions of PTEN, PI3K, AKT, and p-AKT were detected by western blotting post-transfection 48-h. The results showed that *AFAP1-AS1* knockdown promoted the expression of PTEN and inhibited the expressions of PI3K and p-AKT in GH3 and MMQ cells (**Figure 5**).

Discussion

To date, the role of lncRNAs on tumorigenesis and metastasis of pituitary adenoma was poorly understood. In this study, we found that the expression of *AFAP1-AS1* in the pituitary adenoma tissues was considerably higher than its

expression in the adjacent tissues. Thus, *AFAP1-AS1* might act as an oncogene in the pathogenesis of pituitary adenoma.

Previous studies have shown that the expression of *AFAP1-AS1* in non-small-cell lung cancer [14], esophageal squamous cell carcinoma [15], nasopharyngeal carcinoma [16], and ovarian cancer [17] was significantly higher compared to its expression in the adjacent tissues. High expression of *AFAP1-AS1* was associated with tumor progression and poor prognosis in pituitary adenomas. Consistent with the results of previous studies, we found that *AFAP1-AS1* expression was significantly upregulated in the pituitary adenoma tissues compared to its expression in the adjacent tissues. This suggested that the anomalous lncRNA *AFAP1-AS1* may be present as an oncogene in pituitary adenoma. Furthermore, to investigate the effect of *AFAP1-AS1* on the biological functioning of pituitary adenoma cells, we downregulated the expression of *AFAP1-AS1* in the GH3 and MMQ cells and investigated their proliferation, apoptosis, and cell cycle progression. Our results showed that knockdown of *AFAP1-AS1* inhibited the proliferation of GH3 and MMQ cells, promoted apoptosis, and arrested the cell cycle in the G1-to-S phase transition, consistent with the results of previous studies. Previous studies showed similar effect of *AFAP1-AS1* in pancreatic ductal adenocarcinoma [18], hepatocellular carcinoma [19], and colorectal cancer [20].

Finally, we investigated the potential mechanisms by which *AFAP1-AS1* exerted its modulatory effect on tumorigenesis in pituitary adenomas. Recent studies have shown that *AFAP1-AS1* regulated the expression levels of several members of the small GTPase superfamily, and molecules involved in the actin and cytokeratin signaling pathways to promote metastasis in nasopharyngeal carcinoma [21] and lung cancer [22]. A recent study also reported that *AFAP1-AS1* regulated the migration and invasion of hepatocellular carcinoma cells *via* inhibition of the RhoA/Rac2-dependent signaling [23].

AFAP1-AS1 knockdown inhibited the EMT process to inhibit tumor metastasis by promoting the expression of epithelial markers (like E-cadherin) and inhibiting the expression of mesenchymal markers (like N-cadherin and vimentin) in colorectal cancer [24] and gallbladder cancer [25] in previous studies. The above studies are to study the mechanism of *AFAP1-AS1* regulation of tumor metastasis. Guo et al [26] found that *AFAP1-AS1* regulated the PTEN/p-AKT pathway to promote cell proliferation and inhibit apoptosis of gastric cancer cells. The results of the present study suggested that knockdown of *AFAP1-AS1* promoted PTEN expression and inhibited PI3K and p-AKT signaling pathway in GH3 and MMQ cells. The results suggested that knockdown of *AFAP1-AS1* regulated PTEN/PI3K/AKT signaling pathway to inhibit growth and promote apoptosis in GH3 and MMQ cells, which similar with the regulatory mechanism of *AFAP1-AS1* in gastric cancer [26].

In conclusion, *AFAP1-AS1* knockdown inhibited growth and promoted apoptosis in the pituitary adenoma cells which may associate with regulating PTEN/PI3K/AKT signaling pathway. This suggested that *AFAP1-AS1* acts as an oncogene and has therapeutic potential for treating pituitary adenomas in the future.

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Disclosure of conflict of interest

None.

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