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Down-regulation of long non-coding RNA AFAP1-AS1 inhibits tumor growth, promotes apoptosis and decreases metastasis in thyroid cancer

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ABSTRACT

Long non-coding RNAs (lncRNAs), a new type of transcripts, play important roles in various cellular biological processes, involving tumorigenesis. Previous studies showed that lncRNA AFAP1-AS1 was aberrantly expressed in numerous cancers. Nevertheless, we know quite a little about the expression pattern and biological function of AFAP1-AS1 in thyroid cancer. In this study, we adopted the quantitative real-time PCR (qRT-PCR) to detect the expression of AFAP1-AS1 in thyroid cancer tissues. We discovered that expression of AFAP1-AS1 was increased in thyroid cancer tissues. MTT assays elucidated that down-regulation of AFAP1-AS1 could suppress growth of thyroid cancer cells. And the results of flow cytometry analysis indicated knockdown of AFAP1-AS1 induced apoptosis in thyroid cancer. Transwell assay was applied to show decreased cell migration in thyroid cancer as a result of down-regulation of AFAP1-AS1. Hence, our study provided evidence for our hypothesis that AFAP1-AS1 could be a therapeutic target for thyroid cancer.

1. Introduction

Thyroid cancer, which stems from follicular or parafollicular thyroid cells [1], is the most universal endocrine malignancy of the thyroid. The amount of cases has been constantly climbing during the past few decades. The incidence of thyroid cancer has increased more than doubled over the past 30 years in the United States (US) [2]. Thyroid cancer is the eighth most common cancer in China with a rate of 0.368 per 100,000 people [3]. Since the rapid increase in thyroid cancer incidence, thyroid cancer has greatly threatened human beings' health. Therefore, advances in early diagnosis and treatment are urgent to make. As one of the most malignant tumors, thyroid cancers are usually related to specific genetic abnormalities and environmental factors [4]. Radiation, chemotherapy, and surgery are the common treatment methods for thyroid cancer, but they all produce poor satisfaction [5]. So far, due to limited effective biomarkers and diagnostic technology, the molecular and functional mechanisms of thyroid cancer still require further studies.

Recent improvements have revealed a large part of human genome can be transcribed into many noncoding RNAs. Non-coding RNAs are classified into two groups, represented by short RNAs, which are generally less than 200 nt in length and include microRNA (miRNAs); and long non-coding RNAs (lncRNAs), which are more than 200 nt in length [6]. LncRNAs have been associated with a large number of human diseases including cancers, such as hepatocellular carcinoma [7], breast cancer [8] and lung cancer [9]. The abnormal regulation of lncRNAs has already been proved to induce invasion [10], carcinogenesis [11] and metastasis [12] of numerous cancers. And the aberrant expression of lncRNAs has been elucidated in various malignancies, including thyroid cancer.

AFAP1-AS1, a long noncoding RNA, functions as an oncogene in various human cancers. AFAP1-AS1 has been shown to exert biological functions in esophageal adenocarcinoma [13], pancreatic ductal adenocarcinoma [14] and lung cancer [15], but its function in thyroid cancer remains unclear. In this study, we elucidated that AFAP1-AS1 was increased in thyroid cancer tissues and was relevant to patients' survival. And inhibition of AFAP1-AS1 led to growth inhibition, promoted apoptosis and decreased migration in thyroid cells.

2. Materials and methods

2.1. Ethics statement

All experiments with human samples were approved by Nantong Tumor Hospital 226361. Following the Institutional Guidelines, written informed consent was obtained from all patients.

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Table 1
Correlation between AFAP1-AS1 expression and clinical features (n = 36).

Variable	AFAP1-AS1 Expression		P-value
	low	high	
Age			
< 45	7	10	0.187
≥45	12	7	
Gender			
Male	10	7	0.492
Female	9	10	
Pathological Type			
Papillary adenocarcinoma	9	7	0.709
Follicular adenocarcinoma	10	10	
Multicentric Cancer Foci			
Single	7	10	0.187
Multi	12	7	
Lymph Node Metastasis			
Yes	15	6	0.008**
No	4	11	
Surgical Procedure			
Subtotal thyroidectomy	8	9	0.516
Thyroidectomy	11	8	
Tumor Volume			
2~4	7	10	0.187
< 2	12	7	
Clinical Stages			
I-II	12	3	0.006**
III-IV	7	14	

Low/high by the sample mean. Pearson χ^2 test.
** P < 0.05 was considered to be statistically significant.

2.2. Thyroid cancer tissue samples

Fresh samples from FTC tumor tissues (n = 36) and corresponding normal thyroid (NT) tissues adjacent to FTC tumors (n = 36) were obtained from patients with sporadic FTC undergoing surgical resection. Then the samples were snap-frozen in liquid nitrogen and stored at −80 °C. The clinicpathological features of 36 patients are summarized in Table 1.

2.3. Cancer cell line culture

Normal human thyroid follicular epithelial cell Nthy-ori3-1 cell and human thyroid cancer K-1, TPC-1, SW579, FTC133 and XTC-1 purchased from American Type Culture Collection (ATCC) were cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin (Sigma, USA) in the humidified incubator with 5% CO_2 at 37 °C.

2.4. Quantitative real time PCR

Total RNA was extracted from TC tissues of TC patients or TC cells with Trizol Reagent (Qiagen, Hilden). Complementary DNA (cDNA) was synthesized with equal 1 μg of total RNA by using superscript III reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA). One microliter of the cDNA was amplified by real time PCR for AFAP1-AS1 using Bio-Rad iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA) with SYBR Green qPCR Super Mix (Invitrogen, Carlsbad, CA). Relative expression was calculated with the comparative Ct method and normalized against the Ct of GAPDH. The primers for qRT-PCR are as follows: AFAP1-AS1, Forward 5′-TCGCTC AATGGAGTGACGGCA-3′; Reverse 5′-CGGCTGAGACCGCTGAGAA CTT-3′; GAPDH, Forward 5′- CACCCACTCCTCCACCTTTG-3′; Reverse 5′-CCACCACCCTGTTGCTGTAG-3′.

2.5. Cell viability assay

Cell viability was detected using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, after being transfected with siRNA- AFAP1-AS1 and siRNA- AFAP1-AS1 or control siRNA vector for 24 h, thyroid cancer cells were trypsinized and plated in 96-well plates at a density of 5×10^3 cells/well. After 24 h of cultivation, supernatant was abandoned, and 20 μL of MTT was allowed to be added in for another 4 h incubation in RPMI-1640 supplemented with 10% FBS. Consequently, 150 μL of dimethylsulfoxide (DMSO) was mixed with the cells for 10 min. Absorbance of cells in each well was observed at 570 nm under an absorption spectrophotometer (Olympus, Japan) for the cell number calculation.

For further demonstration, CCK-8 (Beyotime Institute of Biotechnology, Jiangsu, China) was also utilized for measuring cell viability. Briefly, we seeded the transfected cells into 96-well plates (2000 cells per well) and followed by cultivation of two days. Whereafter, we added 10 μl CCK-8 solution into each well, then they had to subject to incubation for 2 h at 37 °C. At length, we tested the optical density at 450 nm for it could directly indicated cell viability. To measure the OD value, a microplate reader (Elx800; BioTek Inc., North Brunswick, NJ, USA) was necessary.

2.6. Clonogenic assay

Clonogenic assay was performed to measure the proliferation of thyroid cell lines. In brief, after completion of siRNAs transfection, cells were plated into the 6 wells plate in triplicate and at a cell density of 800 cells per well. Then, the cells were grown in RPMI-1640 with 10% FBS for 14 days. After that, the cells were fixed and stained with Crystal violet, followed with air-dry. Finally, colonies were counted under microscope (IX83, Olympus).

2.7. Cell apoptosis

After transfection with siRNAs, cells were cultured in RPMI-1640 containing 10% FBS for another 48 h, and then, the cells were trypsinized and apoptosis was detected using the Annexin V-FITC Apoptosis Detection Kit (BD, USA). Consequently, cells were pelleted and washed with cold PBS suspended with cold PBS. The TPC and SW579 cells were then treated with Annexin V-propidium iodide (PI) in the dark at room temperature according to the manufacturer's instructions. The cells were kept on ice in the dark and immediately analyzed by flow cytometry (FACSCalibur, BD Biosciences) and the data were analyzed using the Cell Quest software. All the experiments were repeated three times. To actually detect cell apoptosis, we conducted TUNEL staining. This experiment was performed by referring to the report of Li HB et al [16].

2.8. Transwell assay

Cell migration was assessed using Transwell Chamber Cell Culture (10 μm pore membrane, BD Biosciences). A total of 1×10^5 cells in 100 μl of serum-free medium were added to the top chamber of 24-well plates. The bottom well contained growth medium with 20% FBS. Transwell chambers were placed at 37 °C for 48 h. Cells in chamber were fixed with methanol for 30 min and then staining with Crystal violet for 15 min. Migrated cells were finally observed under a microscope and the number was counted with randomly nine field for each experiment.

We evaluated cell invasion ability in accordance with the experimental methods of Wan et al. [17].

2.9. Western blot

Protein expression of N-cadherin, vimentin, E-cadherin, Total-caspase3, Cl-caspase3, Total-caspase9, Cl-caspase9 and GAPDH was