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LncRNA DARS-AS1 regulates microRNA-129 to promote malignant progression of thyroid cancer

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Abstract. – OBJECTIVE: To investigate the expression of long non-coding RNA (lncRNA) DARS-AS1 in thyroid cancer, and to further investigate whether it can promote the development of thyroid cancer by regulating microRNA-129.

PATIENTS AND METHODS: Real-time quantitative polymerase chain reaction (qPCR) was used to detect the level of DARS-AS1 in tumor tissues and paracancerous tissues of 34 thyroid carcinomas. It was also used to analyze the relationship between the expression of DARS-AS1 and the clinical indicators of thyroid cancer and the prognosis of patients. qPCR was used to further verify the expression of DARS-AS1 in thyroid cancer cell lines. The DARS-AS1 knockdown model was constructed using lentivirus in thyroid cancer cell lines. Cell counting kit-8 (CCK-8), cell clone formation, and transwell migration assays were performed to evaluate the effects of DARS-AS1 on the biological function of thyroid cancer cells. Finally, the potential mechanism was explored by using recovery experiments and the interplay between DARS-AS1 and microRNA-129 was further studied.

RESULTS: qPCR results revealed that the level of DARS-AS1 in tumor tissues of thyroid cancer patients was remarkably higher than that in adjacent tissues, and the difference was statistically significant. Compared with patients with low expression of DARS-AS1, patients with high DARS-AS1 expression had a higher incidence of high tumor stage, distant metastasis, and a lower overall survival rate. Besides, compared with NC group, the proliferation and migration ability of shRNA-AS1 expression knockdown group sh-DARS-AS1 was remarkably decreased. qPCR results indicated that there was a negative correlation between the level of microRNA-129 and DARS-AS1 in thyroid cancer tissues. In addition, cell proliferation and migration ability in the microRNA-129 overexpression group were remarkably decreased. The recovery experiment also found that there was a mutual regulation between DARS-AS1 and microRNA-129, which together affected the malignant progression of thyroid cancer.

CONCLUSIONS: DARS-AS1 level in tumor tissues of thyroid cancer was remarkably increased and was correlated with the pathological stage, distant metastasis, and poor prognosis of thyroid cancer. Moreover, DARS-AS1 could promote the proliferation and migration capabilities of thyroid cancer cells by modulating microRNA-129.

Key Words:
DARS-AS1, MiRNA-129, Thyroid cancer, Proliferation, Migration.

Introduction

Thyroid cancer is a type of malignant tumor that originates from thyroid follicular cells or parafollicular cells[1,2]. The incidence of thyroid cancer is on the rise worldwide. In 1990, the number of patients dying from thyroid cancer worldwide was about 24,000. By 2010, this number exceeded 36,000[3,4]. Common thyroid cancers are divided into four categories, namely thyroid papillary carcinoma, thyroid follicular carcinoma, medullary thyroid carcinoma, and thyroid undifferentiated carcinoma[5,6]. About 20% of patients have recurrence and metastasis, and the overall survival rate of patients with thyroid cancer metastasis is remarkably lower than those without metastasis[7]. Currently, the most commonly used treatments for differentiated thyroid cancer include surgical treatment, TSH inhibition therapy, and ^{131}I internal radiation therapy, while chemotherapy and external radiation therapy are rarely

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used for differentiated thyroid cancer. Surgical treatment is the most important and effective treatment for all treatments of thyroid cancer[8,9]. Although the general prognosis of thyroid cancer is generally better after treatment, some patients have recurrence and metastasis, which seriously affect the quality of their life, and even endanger it. They have a double burden on the family's economy and spirit[1,10]. Therefore, further study of its pathogenesis is of great significance for the diagnosis and treatment of diseases.

The Human Genome Project study shows that only about 1% of genes can be transcribed into biologically functional RNA, and the remaining 99% of genes do not have this function of directly encoding proteins, so it is called non-coding RNA without coding protein function (non-coding RNA, ncRNA)[11,12]. However, with the continuous development and progress of science and technology as well as the deepening of understanding of biology, cells, genes, etc., this traditional concept has been broken, and non-coding RNA plays an extremely important role in the complex life activities of organisms[13,14]. Recent works suggest that non-coding RNAs such as long-chain non-coding RNAs and miRNAs play a key role in the development and progression of tumors[15]. Researches have shown that DARS-AS1 plays a role in promoting cancer in some malignant tumors, but the specific mechanism is still unclear, and its role in thyroid cancer remains unknown[16].

Therefore, this study investigated whether long non-coding RNA (lncRNA) DARS-AS1 participated in the progression of thyroid cancer by regulating miRNA-129-mediated proliferation and apoptosis of thyroid cancer.

Patients and Methods

Patients and Thyroid Carcinoma Samples

In this study, 34 pairs of thyroid tissues were selected from surgically treated thyroid cancer cases. The tumor tissues and their corresponding adjacent non-tumor tissues collected from thyroid cancer patients were preserved at -80°C. The collection of clinical specimens was approved by the Ethics Oversight Committee, and patients and their families had been fully informed that their specimens would be used for scientific research, and all participating patients had signed informed consent.

Cell Lines and Reagents

Four PTC cell lines (BHP5-16, TPC, K1, and BHP2-7) and one normal human thyroid epithelial cell line (Nthy-ori 3-1) were obtained from the Chinese Academy of Sciences tumor cell bank (Shanghai, China). Human thyroid cancer cell lines K1 and BHP2-7 were cultured in Dulbecco's Modified Eagle's Medium (DMEM) high glucose medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), penicillin (100 U/mL), and streptomycin (100 μg/mL). All cells were cultured in a 37°C, 5% CO_2 incubator, and cells were passaged to 1% trypsin + EDTA (ethylenediaminetetraacetic acid) for digestion when grown to 80%-90% confluence.

Transfection

DARS-AS1 lentiviral sequence and its corresponding negative control were purchased from Shanghai Jima Company (Shanghai, China). In addition, miRNA-129 overexpression sequence (miRNA-129 mimics) and its negative control were also purchased from Shanghai Jima Company (Shanghai, China). Cells were plated in 6-well plates and grown to a cell density of 70%, then lentiviral transfection was performed according to the manufacturer's instructions, and cells were harvested 48 h later for real-time quantitative polymerase chain reaction (qPCR) analysis and cell function experiments.

Cell Counting Kit-8 (CCK-8) Assay

The cells after 48 h of transfection were harvested and plated into 96-well plates at 2000 cells per well. The cells were cultured for 24 h, 48 h, 72 h, and 96 h respectively. CCK-8 (Dojindo Molecular Technologies, Kumamoto, Japan) reagent was added. After incubation for 2 h, the optical density (OD) value of each well was measured in the microplate reader at 490 nm absorption wavelength.

Colony Formation Assay

The cells after 48 h of transfection were collected, and 200 cells were seeded in each well of a 6-well plate and cultured in complete medium for 2 weeks. The medium was changed after one week, then twice a week. It should be noted that the medium should not be replaced as much as possible in the previous week to avoid cell adhesion. After 2 weeks, the cells were cloned and washed twice with PBS. The cells were fixed in 2 mL of methanol for 20 min. The methanol was aspirated, washed with phosphate buffered saline (PBS), and then stained with 0.1% crystal violet staining solution for 20 min. Then, the cells were washed 3 times with PBS, photographs were counted under a light-selective environment.

Table I. Association of lncRNA DARS-AS1 and miR-129 expression with clinicopathologic characteristics of thyroid carcinoma.

Parameters	Number of cases	DARS-AS1 expression		p-value	miR-129 expression		p-value
		Low (%)	High (%)		High (%)	Low (%)	
Age (years)				0.868			0.588
< 40	14	8	6		5	9	
≥ 40	20	12	8		9	11	
Gender				0.588			0.868
Male	18	9	5		6	8	
Female	16	11	9		8	12	
TNM stage				0.022			0.003
I/II	20	15	5		4	16	
III/IV	14	5	9		10	4	
Lymph node metastasis				0.440			0.493
Negative	22	14	8		10	12	
Positive	12	6	6		4	8	
Distance metastasis				0.010			0.009
Negative	23	17	6		13	10	
Positive	11	3	8		1	10	

Transwell Cell Invasion Assay

The cells after transfection for 48 h were digested, centrifuged and resuspended in medium without FBS to adjust the density to 5 x 10^5 cells/mL. A cell suspension of 200 uL (1 x 10^5 cells) was added to the upper chamber, and 700 uL of a medium containing 20% FBS was added to the lower chamber. The transwell chamber was clipped, washed 3 times with 1 x PBS, and placed in methanol for 15 min cell fixation. After the chamber was stained in 0.2% crystal violet for 20 min, the cells on the upper surface of the chamber were carefully wiped off with water and a cotton swab. The perforated cells stained in the outer layer of the basement membrane of the chamber were observed under the microscope, and 5 fields of view were randomly selected.

QPCR

After the cells were treated accordingly, 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA) was used to lyse the cells, and total RNA was extracted. The initially extracted RNA was treated with DNase I to remove genomic DNA and repurify the RNA. RNA reverse transcription was performed according to the Prime Script Reverse Transcription Kit (TaKaRa, Otsu, Shiga, Japan) instructions, and real-time PCR was performed according to the SYBR® Premix Ex TaqTM (TaKaRa, Otsu, Shiga, Japan) kit instructions. The PCR reaction was performed using the StepOne Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA) system. The following primers were used for qPCR reaction: DARS-AS1: forward: 5'-AGCCAAGGACTGGTCTCTTTT-3', reverse: 5'-CTGTACTGGTGGGAAGAGCC-3'; GAPDH: forward: 5'-CGCTCTCTGCTCCTCCTGTTC-3', reverse: 5'- ATCCGTTGACTCCGA CCTTCAC-3'; miRNA-129: forward: 5'-ACCCAGTGCGATTTGTCA-3', reverse: 5'-ACTGTACTGGAAGATGGACC-3'; U6: forward: 5'-CTCGCTTC GGCAGCACA-3', reverse: 5'-AACGCTTCACGAATTTGCGT-3'. Each sample was subjected to a three-hole repeated experiment and repeated twice. The Bio-Rad PCR instrument was used to analyze and process the data with the software iQ5 2.0 (Bio-Rad, Hercules, CA, USA). The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 genes were used as internal parameters, and the gene expression was calculated by the 2-ΔΔCt method.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 V5.01 software (La Jolla, CA, USA). Statistical differences between the two groups were analyzed using Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). Independent experiments were repeated at least three times for each experiment and data were expressed as mean ± standard deviation. There were three levels of $p<0.05$, $p<0.01$ and $p<0.001$ at the significance level, and $p<0.05$ was considered statistically significant.

Results

LncRNA DARS-AS1 Was Upregulated in Thyroid Carcinoma Tissues and Cell Lines

To determine the role of DARS-AS1 in thyroid cancer, qPCR was used to detect differences in the expression of DARS-AS1 in tumor tissues and adjacent non-tumor tissues of patients with thyroid cancer. The results revealed that DARS-AS1 was elevated in tumor tissues compared with paracancerous tissues in patients with thyroid cancer (Figure 1A). In addition, DARS-AS1 was remarkably higher in thyroid cancer cell lines than Nthy-ori 3-1, and the difference was also statistically significant (Figure 1B).

DARS-AS1 Expression was Correlated with Pathological Staging and Overall Survival in Thyroid Carcinoma Patients

Based on the 34 mRNA expressions of DARS-AS1 in thyroid cancer tissues and paracancerous tissues, we divided DARS-AS1 expression into high expression group and low expression group and analyzed the relationship between the expression of DARS-AS1 and age, sex, pathological stage, lymph node metastasis and distant metastasis of thyroid cancer patients. As shown in Table I, high expression of DARS-AS1 was positively correlated with pathological stage and, distant metastasis of thyroid cancer, but not with age, gender, and lymph node metastasis. In addition, in order to explore the relationship between the expression of DARS-AS1 and the prognosis of patients with thyroid cancer, we collected relevant follow-up data. Kaplan–Meier survival curves revealed that high expression of DARS-AS1 was remarkably associated with poor prognosis of thyroid cancer. The higher the level of DARS-AS1, the worse the prognosis ($p<0.05$; Figure 1C). These results demonstrated that DARS-AS1 expression was correlated with pathological staging and overall survival in thyroid carcinoma patients.

Downregulation of DARS-AS1 Inhibited Cell Growth and Migration

To investigate the function of DARS-AS1 in thyroid cancer, a knockdown DARS-AS1 lentiviral vector was constructed. After transfection of the DARS-AS1 lentiviral vector in the K1 and BHP2-7 cell lines, qPCR experiments were performed to verify the interference efficiency, and the difference was statistically significant (Figure 2A). CCK-8, cell cloning, and transwell assays were used to detect cell proliferation and migration in K1 and BHP2-7 cell lines after knockdown of DARS-AS1. The results revealed that the cell proliferation ability of the DARS-AS1 silencing group (sh-DARS-AS1) was remarkably reduced compared with that of the NC group (Figure 2B-

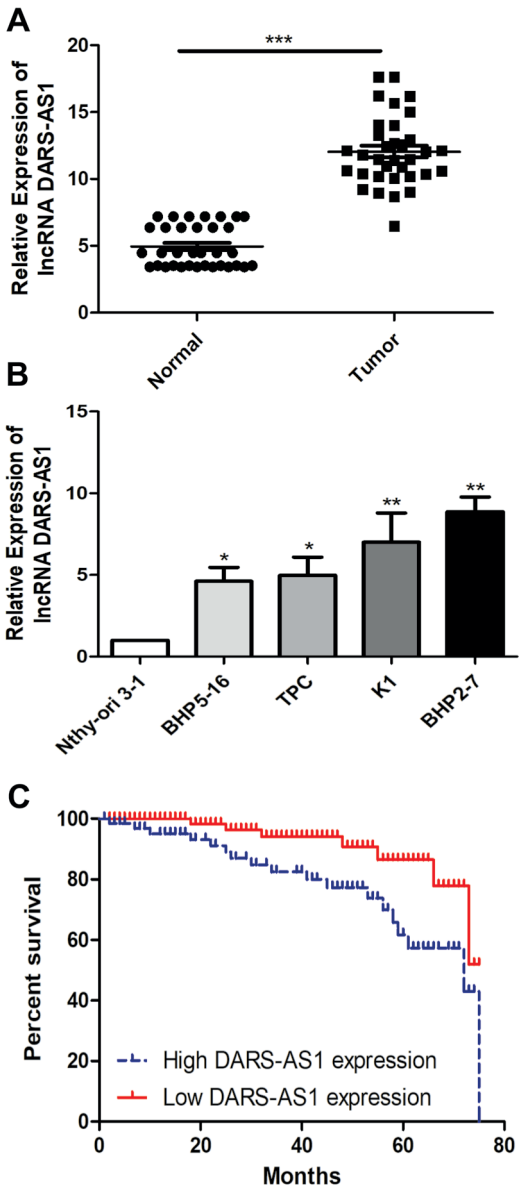

Figure 1. High expression of DARS-AS1 in thyroid cancer tissues and cell lines. **A,** qRT-PCR was used to detect the difference in the expression of DARS-AS1 in tumor tissues and non-tumor tissues adjacent to thyroid cancer patients. **B,** qRT-PCR was used to detect the difference in expression of DARS-AS1 in thyroid cancer cell lines. **C,** Kaplan Meier survival curve of thyroid cancer patients based on DARS-AS1 expression; the prognosis of patients with high expression was significantly worse than that of the bottom expression group. Data are mean ± SD, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

2C). In addition, the transwell assay revealed a significant decrease in cell migration ability in the DARS-AS1 silencing group compared to that of the NC group (Figure 2D), suggesting that downregulation of DARS-AS1 inhibited cell growth and migration.

MicroRNA-129 Was Downregulated in Thyroid Carcinoma Tissues and Cell Lines

QPCR experiments revealed that the level of microRNA-129 was remarkably decreased in tumor tissues of thyroid cancer patient compared with paracancerous tissues, and the difference was statistically significant (Figure 3A). In addition, compared with Nthy-ori 3-1, microRNA-129 was remarkably downregulated in thyroid cancer cells, and the difference was also statistically significant (Figure 3B), suggesting that microRNA-129 may play a tumor suppressor gene in thyroid cancer. Then the expression of DARS-AS1 and microRNA-129 was detected by qPCR. The results revealed that the expression of DARS-AS1 and microRNA-129 were negatively correlated in thyroid cancer tissues (Figure 3C). In addition, we analyzed the pathology and prognosis of microRNA-129 in thyroid cancer. As shown in Table I, low expression of microRNA-129 was positively correlated with pathological stage and distant metastasis of thyroid cancer, but not with age, gender, and lymph node metastasis. In addition, the Kaplan–Meier survival curve revealed that low expression of microRNA-129 was remarkably associated with poor prognosis of thyroid cancer, and the lower the level of microRNA-129, the worse the prognosis (Figure 3D).

Upregulation of MicroRNA-129 Inhibited Cell Growth and Migration

To investigate the function of microRNA-129 in thyroid cancer, a microRNA-129 mimics was constructed. After transfection of the microRNA-129 lentiviral vector in the K1 and BHP2-7 cell lines, qPCR experiments were performed to verify the interference efficiency, and the difference was

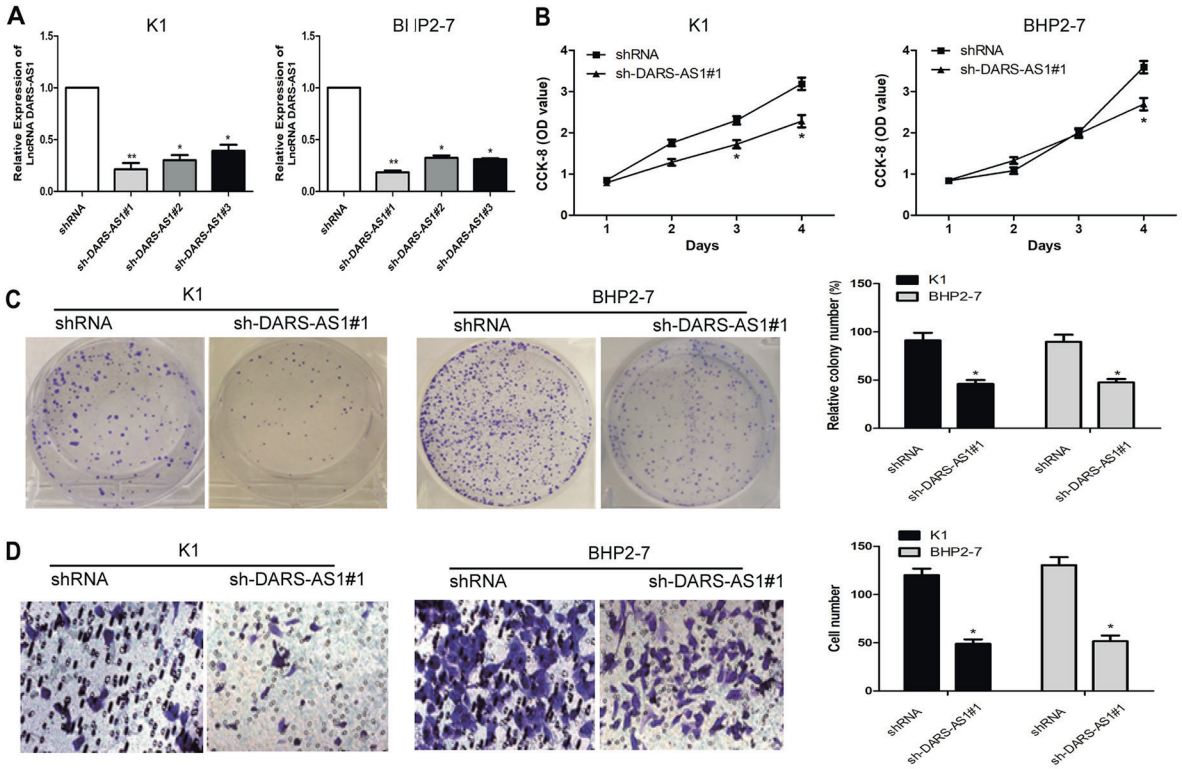

Figure 2. Silencing DARS-AS1 inhibits proliferation and migration of thyroid cancer cells. **A,** qRT-PCR verified the interference efficiency of DARS-AS1 after transfection of the DARS-AS1 knockout vector in the K1 and BHP2-7 cell lines. **B,** The CCK-8 assay detects the effect of silencing DARS-AS1 on the proliferation of thyroid cancer cells in K1 and BHP2-7 cell lines. **C,** Cell clone formation assay detects the effect of silencing DARS-AS1 on proliferation of thyroid cancer cells in K1 and BHP2-7 cell lines. **D,** Transwell experiments further indicated the ability of DARS-AS1 to silence thyroid cancer cells after silencing DARS-AS1 in K1 and BHP2-7 cell lines (magnification: 20X). Data are mean ± SD, *p<0.05.

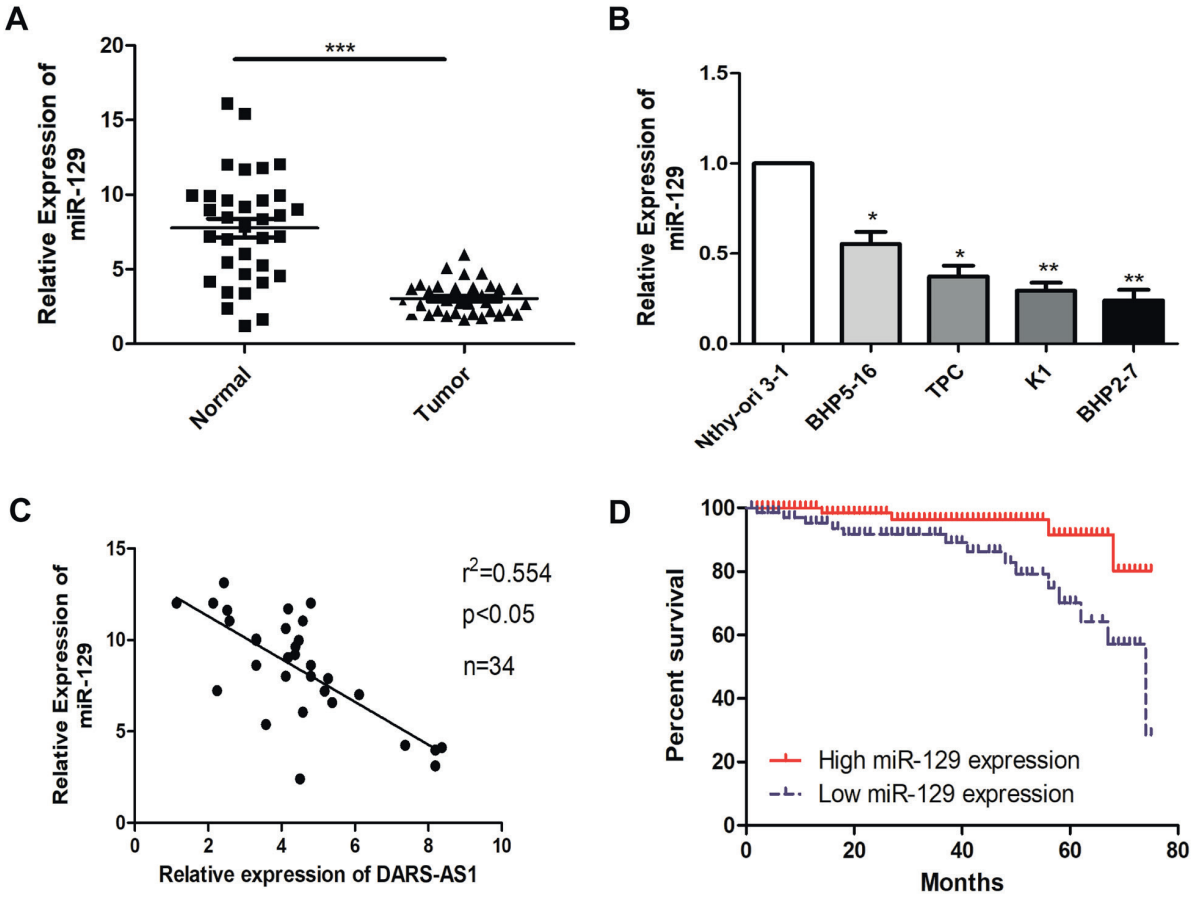

Figure 3. Direct targeting of miR-129 by DARS-AS1. **A,** qRT-PCR was used to detect the difference in expression of miR-129 in tumor tissues and adjacent non-tumor tissues in patients with thyroid cancer. **B,** qRT-PCR was used to detect the mRNA expression level of miR-129 in thyroid cancer cell lines. **C,** There was a significant negative correlation between the expression levels of DARS-AS1 and miR-129 in thyroid cancer tissues. **D,** Kaplan Meier survival curve of thyroid cancer patients based on miR-129 expression. Data are mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

statistically significant (Figure 4A). Cell proliferation and migration were measured by CCK-8, cell cloning and transwell assay after overexpression of microRNA-129 in K1 and BHP2-7 cell lines. The results revealed that the cell proliferation ability of the microRNA-129 overexpression group microRNA-129 mimics was remarkably reduced compared to those of the NC group (Figure 4B-4C). In addition, the transwell assay revealed a significant decrease in cell migration ability after microRNA-129 overexpression (Figure 4D). In sum, these results revealed that upregulation of microRNA-129 inhibited cell growth and migration.

DARS-AS1 Modulated MicroRNA-129 Expression in Thyroid Carcinoma

To further explore the ways in which DARS-AS1 promoted the malignant progression of thyroid cancer, we found a possible relationship between DARS-AS1 and microRNA-129 through relevant bioinformatics analysis. MicroRNA-129 was knocked down in DARS-AS1 silencing cell lines in thyroid cancer cells to investigate whether it can promote the development of thyroid cancer (Figure 5A). Subsequently, we used CCK-8, cell cloning and transwell experiments to evaluate that knockdown of microRNA-129 reversed the effect of knockdown of DARS-AS1 on proliferation and migration of thyroid cancer cells (Figure 5B-5D), suggesting that DARS-AS1 could modulate microRNA-129 to promote the proliferation and migration capabilities of thyroid carcinoma.

Discussion

Long-chain non-coding RNAs (lncRNAs) are a class of RNAs with more than 200 nucleotides in length and no protein-coding function[11,12]. Long-

chain non-coding RNA can play an important regulatory role in cell proliferation, migration, and invasion at epigenetic, transcriptional, and post-transcriptional levels, thereby affecting the expression of coding genes through various mechanisms such as DNA methylation and demethylation, RNA interference, histone modification, chromatin remodeling, and imprinted genes[13]. In addition, abnormal lncRNAs can be found in malignant tumors, suggesting that lncRNAs play the role of tumor suppressor genes or oncogenes in the development and progression of tumors[12-14]. Scholars[14,15] have found that lncRNA plays an important role in the development and progression of thyroid cancer. In this study, the thyroid cancer knockdown cell line established by lentivirus was combined with bioinformatics to analyze the difference in expression of long non-coding RNA between thyroid cancer cell lines K1 and BHP2-7, and DARS-AS1 was selected from it. DARS-AS1 was studied as a candidate for lncRNA related to malignant progression of thyroid cancer, and finally, the relationship between DARS-AS1 and the development of thyroid cancer was determined. It was shown that upregulation of DARS-AS1 can promote the malignant progression of thyroid cancer. Through tissue verification, we found that the expression of DARS-AS1 in thyroid cancer tissues was remarkably higher than that in adjacent tissues, and was positively correlated with tumor stage, distant metastasis and poor prognosis of thyroid cancer. Therefore, we

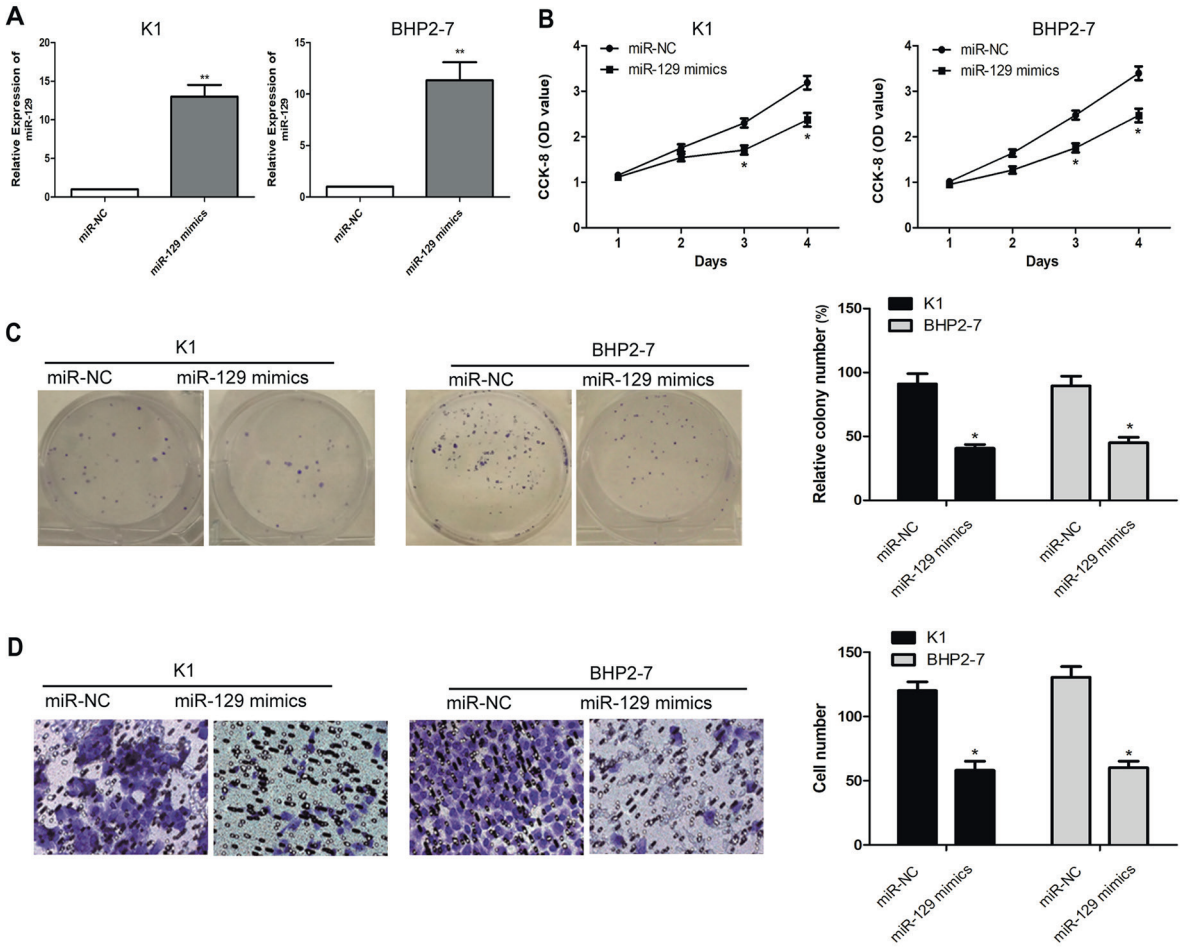

Figure 4. Overexpression of miR-129 inhibits proliferation and migration of thyroid cancer cells. **A,** qRT-PCR verified the interference efficiency of DARS-AS1 after transfection of the miR-129 overexpression vector in K1 and BHP2-7 cell lines. **B,** The CCK-8 assay detects the effect of miR-129 overexpressing K1 and BHP2-7 cell lines on thyroid cancer cell proliferation. **C,** Cell clone formation assays examined the effect of overexpression of miR-129 on proliferation of thyroid cancer cells in K1 and BHP2-7 cell lines. **D,** Transwell experiments further detected the ability of overexpressing miR-129 to mimic thyroid cancer cells after overexpression of miR-129 in K1 and BHP2-7 cell lines (magnification: 20X). Data are mean ± SD, *p<0.05.

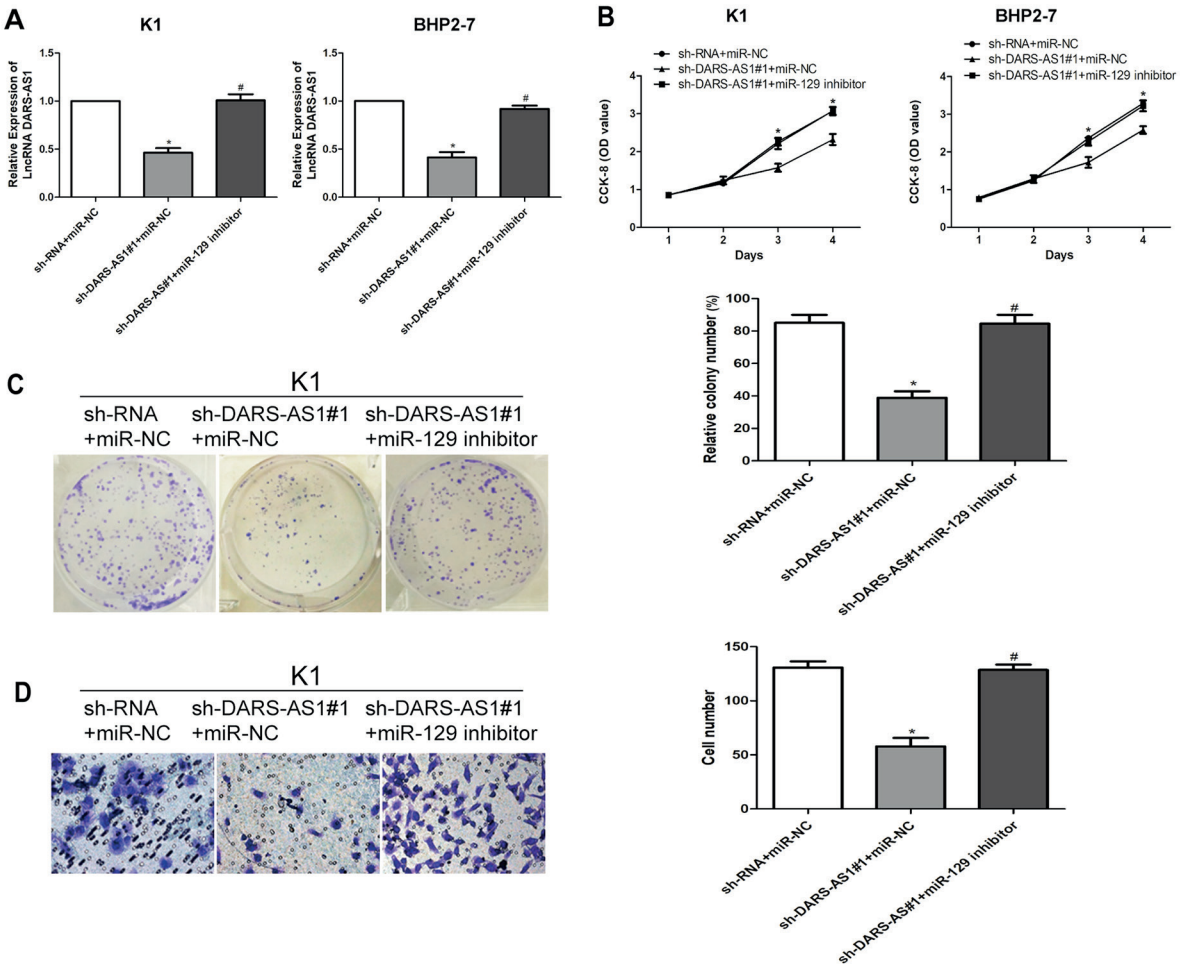

Figure 5. DARS-AS1 regulates the expression of miR-129 in thyroid cancer tissues and cell lines. **A,** DARS-AS1 expression levels in DARS-AS1 and miR-129 co-transfected cell lines were detected by qRT-PCR. **B,** CCK-8 assay detects the role of DARS-AS1 and miR-129 in the regulation of thyroid cancer cell proliferation after co-transfection. **C,** Cell clone formation assay detects the proliferation of thyroid cancer cells after co-transfection of DARS-AS1 and miR-129. **D,** The transwell assay further confirmed the ability of silencing DARS-AS1DARS-AS1 and miR-129 to regulate thyroid cancer cell migration after co-transfection (magnification: 20X). Data are mean ± SD, *$p<0.05$.

believe that DARS-AS1 may play a role in promoting thyroid cancer.

In order to further study the molecular mechanism of DARS-AS1 in the development of thyroid carcinoma, *in vitro* cell experiments were performed. To observe the effect of DARS-AS1 on the proliferative capacity of thyroid cancer cells, we performed CCK-8 assay, cell clone formation, and transwell assay. We found that silencing DARS-AS1 in K1 and BHP2-7 cell lines remarkably attenuated the cell proliferation, colony formation and migration ability, indicating that shi-DARS-AS1 can inhibit the proliferation and migration of human thyroid cancer cells. In addition, we also explored the molecular mechanism of microRNA-129 in the development of thyroid cancer and found that microRNA-129 was overexpressed in thyroid cancer cells. Cell clone formation and migration assay showed that microRNA-129 can inhibit the proliferation and migration of thyroid cancer cells.

Authors[17,18] have shown that long-chain non-coding RNA can be used as a competitive endogenous RNA (ceRNA) to compete with other RNA transcripts for the same miRNA in addition to the regulation of gene expression, in order to achieve mutual communication and regulation. At present, functional studies on competitive endogenous RNA suggest that miRNAs and long-chain non-coding RNAs can be mutually regulated by

competitive binding to corresponding miRNA response elements, thereby effectively controlling the subsequent post-transcriptional regulation of miRNAs[18-20]. Therefore, miRNAs and long-chain non-coding RNAs can affect the expression of target genes through mutual regulation, and thus participate in the development of tumors[21]. In this project, we used bioinformatics analysis to predict the target miRNA of lncRNA and found that miRNA-129 may interact with DARS-AS1. The results of this experiment suggest that miRNA-129 was less expressed in tumor tissues of thyroid cancer patients than in adjacent tissues and that miRNA-129 can inhibit proliferation and migration in thyroid cancer cells. To further investigate the regulation of DARS-AS1 and miRNA-129 expression in thyroid cancer cell lines, we found that the levels of DARS-AS1 and microRNA-129 were just negatively correlated. In addition, the recovery experiment found that knockdown of miRNA-129 can counteract the role of silencing DARS-AS1 in thyroid cancer cell lines. Therefore, combined with the above findings, it is suggested that DARS-AS1 can inhibit the expression of miRNA-129, thereby promoting the proliferation and migration of thyroid cancer cells.

Conclusions

The expression of LncRNA DARS-AS1 was remarkably increased in thyroid cancer, which was closely associated with pathological stage, distant metastasis and poor prognosis of thyroid cancer. In addition, DARS-AS1 may promote the malignant progression of thyroid cancer by regulating miRNA-129.

Conflicts of interest

The authors declare no conflicts of interest.

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