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Feasibility study of lncRNA DHRS4-AS1 sponge miR-222-3p in the diagnosis of thyroid cancer

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Abstract

Introduction: Thyroid cancer is a commonly occurring malignant tumour within the endocrine system, the incidence of which has been increasing steadily in our country. It has been the focus and direction of research in recent decades to continuously explore the diagnostic markers and molecular mechanisms of thyroid cancer and provide new possibilities for the healing of patients. In this study, lncRNA DHRS4-AS1 was identified as the research target, and the regulatory function of abnormal expression of DHRS4-AS1 on thyroid cancer was discussed.

Material and methods: Thyroid cancer (116) and non-cancer normal (82) tissue samples were collected in this paper, and the expression of DHRS4-AS1 and miR-222-3p in tissues and cells were evaluated by RT-qPCR. CCK-8 and flow cytometry were used to detect cell survival status. The mechanism of DHRS4-AS1 sponge miR-222-3p was analysed by dual-luciferase reporter gene detection.

Results: In the present study, DHRS4-AS1 was down-regulated in both thyroid tissue and cell samples, while miR-222-3p expression was elevated. The ROC curve reflected the diagnostic value of DHRS4-AS1 in thyroid cancer [area under the curve (AUC) = 0.887, sensitivity = 76.7%, specificity = 95.1%]. DHRS4-AS1 regulates the development of thyroid cancer by targeting miR-222-3p. In addition, *in vitro* experiments demonstrated that overexpression of DHRS4-AS1 (pcDNA3.1-DHRS4-AS1) inhibited the proliferation of thyroid cancer cells and promoted cell apoptosis, while down-regulating the level of miR-222-3p.

Conclusions: DHRS4-AS1 acts as a miR-222-3p sponge in thyroid cancer, and overexpression of DHRS4-AS1 down-regulates cell proliferation and promotes cell apoptosis. These findings demonstrate the potential of DHRS4-AS1 as a diagnostic factor for thyroid cancer. **(Endokrynol Pol 2024; 75 (5): 494–500)**

Keywords: *thyroid cancer; lncRNA DHRS4-AS1; miR-222-3p; diagnosis; proliferation; apoptosis*

Introduction

Thyroid cancer is a specific thyroid nodule that occurs in thyroid follicles or parafollicular epithelial cells and accounts for approximately 2.5% of all cancers and 90% of endocrine tumours [1, 2]. Among them, more than 90% of thyroid cancer patients have a good prognosis and a high survival rate [3, 4]. With the trend of increasing incidence rate and mortality of thyroid cancer in many countries and regions year by year, thyroid cancer has become one of the most concerned endocrine system tumours [5]. Currently, it is generally believed that thyroid cancer is the result of multiple factors such as environmental factors, genetic factors, ionising radiation, growth factors, psychosocial factors, and mental conditions [6–8]. However, the exact aetiology of thyroid cancer has not been determined, and the molecular mechanism of disease progression is still unclear. Therefore, timely prevention, diagnosis, and treatment of patients still have a long way to go.

LncRNAs refer to a class of RNA transcripts larger than 200 bp, which are mostly considered as by-products of the transcription process [9]. With the deepening of research, a large amount of evidence has identified that

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lncRNAs participate in cell differentiation and regulate the pathogenesis of many diseases, including tumours [10]. Several lncRNAs, including RUNDC3A-AS1, LINC02454, and CATIP-AS1, have been reported to be engaged in the pathogenesis of thyroid cancer [11–13]. What is remarkable is that lncRNA DHRS4-AS1 is a natural antisense transcript of the DHRS4 gene located on chromosome 14q11.2 [14, 15]. In endometriosis, DHRS4-AS1 has been outlined to attenuate cell biological function by targeting miR-139-5p [16]. In the study of clear cell renal cell carcinoma, down-regulated expression of DHRS4-AS1 has been determined to affect the prognosis of patients, which can be used as a tumour suppressor in the treatment of patients [14]. In hepatocellular carcinoma, DHRS4-AS1 acts as a potential therapeutic marker to inhibit tumour development by regulating the miR-522-3p/SOCS5 axis [17]. Based on this, we speculate that DHRS4-AS1 may also play a role in the metastasis and development of thyroid cancer as a sponge of miRNA.

This study detected the level of DHRS4-AS1 in thyroid cancer, explored the regulatory mechanism of DHRS4-AS1 combined with miRNA in the development of thyroid cancer, and expounded the potential diagnostic value of DHRS4-AS1, providing new guidance for the healing of thyroid cancer.

Material and methods

Acquisition of tissue samples

The included tissue samples consisted of 116 thyroid cancer tissues and 82 non-cancer normal tissues obtained from patients undergoing thyroidectomy at **XXX Hospital**. The obtained tissue samples were briefly processed (residual blood was washed away with PBS) and stored at –80°C. All patients were diagnosed with thyroid cancer and other complications were excluded, and the patients had not received other anticancer treatments before participating in the study. The procedure was carried out with the knowledge of the patients and the approval of the hospital.

Experimental cells

The cell medium was RPMI-1640 with 10% foetal bovine serum and 1% penicillin streptomycin solution, and the cell culture condition was 37°C in an incubator containing 5% CO_2. Experimental thyroid cells (B-CPAP, FTC-133, and TPC-1) and control normal thyroid epithelial cells (Nthy-ori 3-1) were derived from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China).

To investigate the effect of overexpression of DHRS4-AS1 on biological function of pancreatic cancer cells, the sequences of DHRS4-AS1 were transfected into pcDNA3.1 plasmid (Invitrogen, USA) after Lipofectamine 2000 addition to construct pcDNA3.1-DHRS4-AS1. The transfection efficiency was verified by RT-qPCR. By the same method, pcDNA3.1-DHRS4-AS1-mimic NC and pcDNA3.1-DHRS4-AS1-miR-222-3p mimic were constructed through pcDNA3.1 plasmid.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay

TRIzol (Invitrogen, USA) reagent was added to the broken tissue and cell samples for RNA extraction. After confirming RNA purity and concentration, RNA was reverse transcribed into cDNA according to the instructions of the PrimeScript RT Mix kit (TaKaRa, Japan). Then, with the participation of SYBR Green PCR Kit (TaKaRa, Japan), the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) system was configured using cDNA as a template, and the reaction was run in the ABI 7500 system (Applied Biosystems, USA). The data obtained were treated by $2^{-\Delta\Delta Ct}$, and GAPDH and U6 were used as internal controls. The primer sequences were as follows: DHRS4-AS1 F-5′-GGAGGCTGAGGCAGGAG-3′, R-5′-GCTAGTCTGGTCACCTCTG-3′; miR-222-3p F-5′-CGGCACGGGCCGAGGC-3′, R-5′-AGTGCAGGGTCCGAGGTATT-3′; GAPDH F-5′-GCACCGTCAAGGCTGAG-3′, R-5′-TGAAGACGCCAGTGGA-3′; U6 F-5′-CTCGCTTCGGCAGCACA-3′, R-5′-CGCTTCACGAAATTTGCGT-3′.

Luciferase activity assay

After DHRS4-AS1 binding factors were predicted by bioinformatics software, according to the binding sites between DHRS4-AS1 and miR-222-3p, wild and mutant type of DHRS4-AS1 (WT-DHRS4-AS1 and MUT-DHRS4-AS1) were constructed by inserting the amplified fragment into pmirGLO plasmid. Then, miR-222-3p mimic was co-transfected into TPC-1 cells, and the luciferase activity of cells was detected by dual-luciferase reporting assay system (Promega Corporation) to understand the interaction between DHRS4-AS1 and miR-222-3p.

Cell count kit 8 (CCK-8) assay

Cell Counting Kit-8 (CCK-8; Dojindo, Japan) was selected to examine the proliferation of thyroid cancer cells. The transfected cells were transferred to 96-well plates, and CCK-8 solution was added in sequence within the following 4 days of culture. After continued incubation for 2 hours, the absorbance value at 450 nm was measured in an enzyme label to evaluate the number of cells.

Apoptosis assay

With the instructions of the Annexin V-FITC/PI apoptosis detection kit (Solarbio, China), the cells were induced to apoptosis, and the level of apoptosis was detected according to the following operations. Specifically, the transfected thyroid cancer cells were collected and resuspended in annexin V binding buffer, followed by Annexin V-FITC and PI reagents and treated for 15 min in the dark environment. Finally, the apoptotic cells were monitored and analysed by flow cytometry.

Statistical analysis

The differences between the 2 groups and the above groups were selected by Student's *t* test or one-way ANOVA analysis. In addition, the receiver operating curve (ROC) reflected the potential of DHRS4-AS1 in the diagnosis of thyroid cancer. The correlation analysis between clinical features of thyroid cancer patients and DHRS4-AS1 expression level was confirmed by chi-square analysis. Three sets of parallel assays were set up for each set of samples with at least 3 replications. p value less than 0.05 was considered significant.

Results

Abnormal low expression of DHRS4-AS1 in thyroid cancer

According to the ENROCI project (data were downloaded from The Cancer Genome Atlas [TCGA] project), DHRS4-AS1 decreased in thyroid cancer samples compared with normal samples (Fig. 1A). Based on this, 116 thyroid cancer tissues and 82 normal tissues were collected, and the expression of DHRS4-AS1

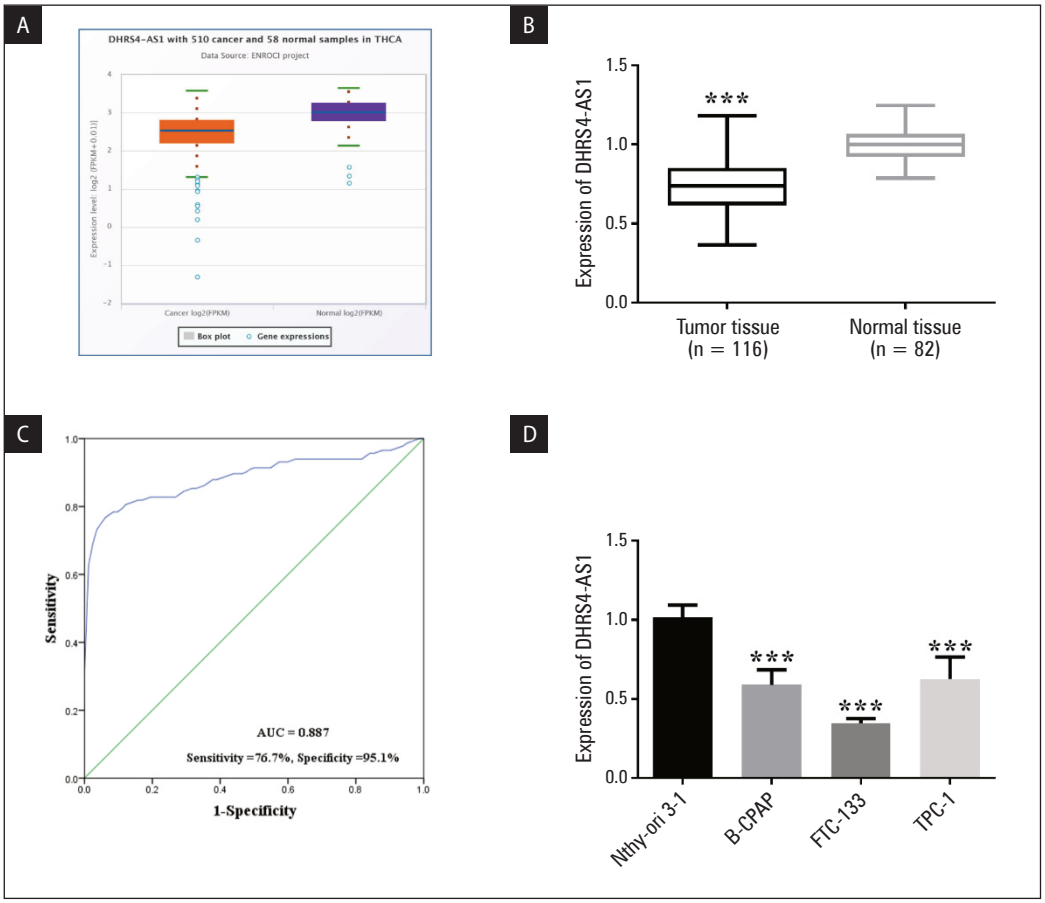

Figure 1. *Expression and diagnostic potential of DHRS4-AS1 in thyroid gland.* **A.** *The Cancer Genome Atlas (TCGA) database showed that DHRS4-AS1 decreased in thyroid cancer samples;* **B.** *DHRS4-AS1 was significantly lower in thyroid carcinoma than in normal tissue;* **C.** *Receiver operating characteristic (ROC) curve reflected the diagnostic value of DHRS4-AS1 in thyroid cancer (area under the curve (AUC) = 0.887, sensitivity = 76.7%, specificity = 95.1%);* **D.** *The level of DHRS4-AS1 was lower in thyroid cancer cells B-CPAP, FTC-133 and TPC-1 than in normal thyroid epithelial cells Nthy-ori 3-1 (***p < 0.001)*

was detected by RT-qPCR method. DHRS4-AS1 was down-regulated in thyroid cancer tissues as shown in Figure 1B. Taking the mean expression of DHRS4-AS1 in thyroid cancer tissues as the critical value, the included patients could be divided into a low DHRS4-AS1 expression group (n = 60) and a high DHRS4-AS1 expression group (n = 56). Table 1 illustrates the relationship between DHRS4-AS1 and clinicopathologic parameters of patients with thyroid cancer. It can be seen that tumour size (p = 0.017), TNM stage (p = 0.032), and lymph node metastasis (p = 0.029) are significantly correlated with DHRS4-AS1 expression. In Figure 1C, the ROC curve reflected that the AUC was 0.887 in a comparison between thyroid cancer tissue samples and normal control samples, the sensitivity was 76.7%, and the specificity was 95.1%, indicating the high diagnostic value of DHRS4-AS1 in thyroid cancer. In addition, the DHRS4-AS1 level in thyroid cancer cells B-CPAP, FTC-133, and TPC-1 also showed a downward trend compared with normal thyroid epithelial cells (Nthy-ori 3-1) by detecting the level of DHRS4-AS1 in cells, which further confirmed the previous results in Figure 1D.

DHRS4-AS1 acts as a sponge for miR-222-3p

To further explore the regulation and biological function of DHRS4-AS1 abnormal expression on thyroid cancer, pcDNA3.1 plasmid was used to construct pcDNA3.1-DHRS4-AS1. Figure 2A illustrates that pcDNA3.1-DHRS4-AS1 can effectively increase the content of DHRS4-AS1 in TPC-1 and FTC-133 cells. Subsequently, we predicted the potential combination of DHRS4-AS1 with miR-222-3p through Starbase website, as shown in Figure 2B. The detection of miR-222-3p expression in tissue samples showed that, unlike the down-regulation of DHRS4-AS1 in thyroid cancer tissues, miR-222-3p expression was increased in tumour tissues (Fig. 2C). Transfection of pcDNA3.1-DHRS4-AS1 in TPC-1 and FTC-133 cells notably decreased the content of miR-222-3p, as can be seen in Figure 2D. Furthermore, luciferase reporter analysis, as shown in Figure 2E, confirmed

Table 1. *Association between DHRS4-AS1 and clinicopathological parameters of thyroid cancer*

Parameters	LncRNA DHRS4-AS1 expression			p
	n =116	Low (n = 60)	High (n = 56)	
Age (years)				
≤ 55	67	35	32	0.897
> 55	49	25	24	
Sex				
Male	67	34	33	0.805
Female	49	26	23	
Tumour size				
≤ 1 cm	81	36	45	0.017
> 1 cm	35	24	11	
TNM stage				
I, II	87	40	47	0.032
III, IV	29	20	9	
Extra thyroidal extension				
No	83	41	42	0.426
Yes	33	19	14	
Lymph node metastasis				
No	94	44	50	0.029
Yes	22	16	6	

TNM — tumour–node–metastasis

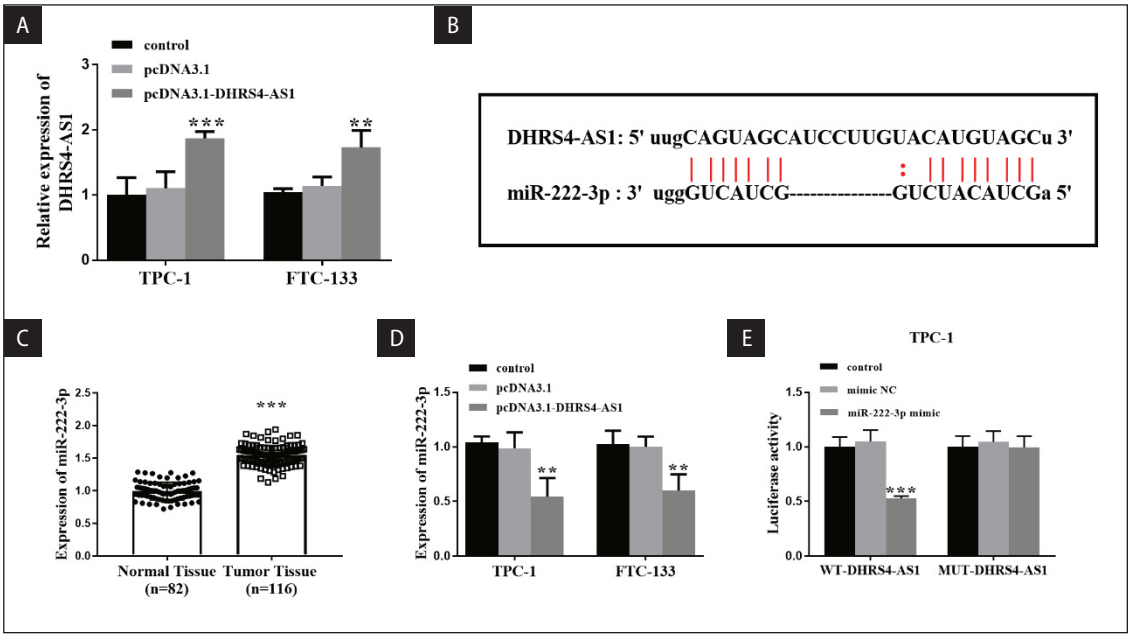

Figure 2. *DHRS4-AS1 acts as a sponge for miR-222-3p.* **A.** *The content of DHRS4-AS1 was increased after transfection of pcDNA3.1-DHRS4-AS1 in TPC-1 and FTC-133 cells;* **B.** *There are binding sites between DHRS4-AS1 and miR-222-3p;* **C.** *Increased expression of miR-222-3p in thyroid cancer tissue;* **D.** *miR-222-3p expression in thyroid cancer cells decreased after transfection of pcDNA3.1-DHRS4-AS1;* **E.** *The luciferase activity of TPC-1 cells transfected with WT-DHRS4-AS1 was lower in miR-222-3p mimic than in control group (**p < 0.01, ***p < 0.001)*

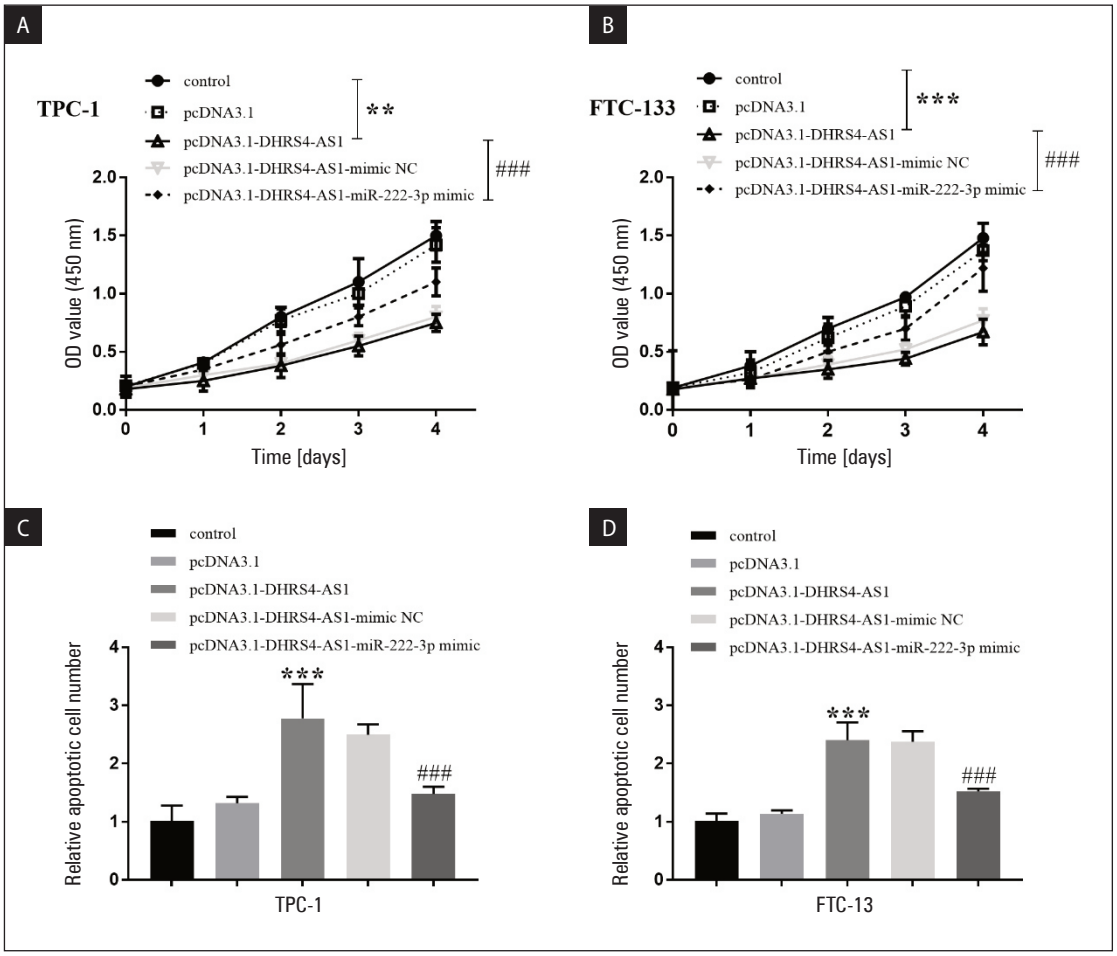

Figure 3. *DHRS4-AS1 targets miR-222-3p to regulate the proliferation and apoptosis of thyroid cancer cells.* **A/B.** *TPC-1 and FTC-133 cells transfected with pcDNA3.1-DHRS4-AS1 showed decreased proliferative ability compared with the control group, while co-transfected with pcDNA3.1-DHRS4-AS1-miR-222-3p mimic reversed this inhibitory effect;* **C/D.** *High expression of DHRS4-AS1 increased the rate of apoptosis of thyroid cancer cells, while transfection of miR-222-3p mimic restored the apoptosis rate. (**$p < 0.01$, ***$p < 0.001$ vs. control group; ###$p < 0.001$ vs. pcDNA3.1-DHRS4-AS1 group)*

that miR-222-3p mimic decreased luciferase activity of WT-DHRS4-AS1 in TPC-1 cells.

Cell proliferation and apoptosis experiment in vitro

In *in vitro* cell experiments, we explored the regulation of DHRS4-AS1 sponge miR-222-3p on thyroid cancer cells. In Figure 3A, transfection of pcDNA3.1-DHRS4-AS1 markedly inhibited the proliferation of TPC-1 cells, and this inhibition was eliminated after co-transfection of pcDNA3.1-DHRS4-AS1-miR-222-3p mimic. Similarly, this recovery effect was also reflected in FTC-133 cells (Fig. 3B). As shown in Figure 3C, the apoptosis level of TPC-1 cells was sharply elevated after transfection of pcDNA3.1-DHRS4-AS1. Compared with that, the apoptosis level of TPC-1 cells was decreased after co-transfection of pcDNA3.1-DHRS4-AS1-miR-222-3p mimic. The same is true in Figure 3D, overexpression of DHRS4-AS1 increased the level of apoptosis in FTC-133 cells, while the involvement of miR-222-3p mimic could counteract the level of apoptosis.

Discussion

Thyroid cancer, as certified by cancer authorities in Korea and the United States, is the fastest growing type of cancer in the past few decades, the global incidence of which has also increased about 3 fold and is still increasing at an annual rate of 3.6%, with a distinctly higher incidence in women than in men [18–21]. Most thyroid cancer patients with a good prognosis after surgical treatment, combined with radiotherapy or targeted therapy, and can survive for a long time after surgery [22]. However, thyroid cancer patients have no special clinical symptoms in the early stage, and the cause of the disease has not been identified. Therefore, the development of accurate and effective diagnostic markers will be helpful for the diagnosis and future treatment of thyroid cancer patients.

Previous reports suggest that the abnormal expression of lncRNA is involved in the pathogenesis of thyroid cancer. For example, in the research results of Zhang et al., CALML3A-S1 was down-regulated in papillary thyroid carcinoma (PTC), and overexpression of CALML3-AS1 inhibited PTC cell activity both *in vitro* and *in vivo* [23]. Pan et al. confirmed that LINC01089 expression in thyroid cancer tissues was significantly reduced and correlated with the tumour stage and lymph node metastasis of patients, suggesting that LINC01089 has great potential as a therapeutic target for thyroid cancer [24]. Down-regulation of lncRNA DANCR is closely related to T grade and TNM stage of patients, which has potential as a diagnostic factor for PTC, as discovered by Zhang et al. [25]. In our study, DHRS4-AS1 was shown to be lower in the thyroid cancer sample group (tissue and cell) than in the normal control group, and it was correlated with the tumour size, TNM stage, and lymph node metastasis of patients. The ROC curve clarified the diagnostic function of DHRS4-AS1 in thyroid cancer, which was consistent with the above conclusions. Meanwhile, low DHRS4-AS1 content in non-small cell carcinoma, as demonstrated by Yan et al. in 2020, was involved in the regulation of cell stemness [26]. In addition, Zhou et al. found DHRS4-AS1 expression in hepatocellular carcinoma cells to be lower than normal [17].

As a competitive endogenous RNA, lncRNA plays a sponge adsorption role in inhibiting the expression of miRNA [27, 28]. In this study, the complementary binding sites between DHRS4-AS1 and miR-222-3p were identified by Starbase software prediction and luciferase activity experiments. MiR-222-3p was elevated in thyroid cancer, and overexpression of DHRS4-AS1 decreased the expression of miR-222-3p. Similarly, the level of miR-222-3p was up-regulated in patients with carotid artery stenosis, and miR-222-3p in cells could negatively regulate lncRNA GAS5 and affect luciferase activity [29]. In the current study, overexpression of DHRS4-AS1 inhibited the proliferation of TPC-1 and FTC-133 cells and increased the apoptosis of cells at the same time, thus exhibiting a tumour suppressive effect. However, transfection with pcDNA3.1-DHRS4-AS1-miR-222-3p mimic negated the effect of overexpression of DHRS4-AS1 on the progression of thyroid cancer. Similarly, Li et al. showed that GAS5 was down-regulated in thyroid cancer, while the up-regulation of miR-362-5p reversed the effect of lncRNA GAS5 on thyroid cancer cells [30]. Qi et al. reported that overexpression of miR-515-5p offset the inhibition of the activity of CATIP-AS1 on thyroid cancer cells and the induction of apoptosis [13]. Importantly, existing studies have demonstrated the efficacy of miR-222-3p in thyroid cancer. For example, the high level of miR-222-3p regulates the biological function of SLC4A4 to promote PTC cells [31]. The up-regulated expression of miR-222-3p with high sensitivity and specificity can be considered as an independent diagnostic and prognostic factor for PTC [32]. All the above elucidated the possibility of DHRS4-AS1 regulating the progression of thyroid cancer by directly targeting miR-222-3p.

There are some shortcomings in this study. First, the study is still in the preliminary stage of exploration, so the sample size included is limited. Second, the study was limited to the assessment of cellular activity *in vitro* and lacked the confirmation of *in vivo* experiments. Follow-up work could be based on this foundation to further evaluate the relevant pathological mechanisms and pharmacological interventions of DHRS4-AS1 in thyroid cancer, with a view to bringing feasible therapeutic ideas for patients.

Conclusions

In summary, this study demonstrated that DHRS4-AS1 significantly decreased in thyroid cancer, and it suggested that high expression of DHRS4-AS1 sponge miR-222-3p inhibited cell proliferation and promoted cell apoptosis, thus effectively controlling the development of thyroid cancer. DHRS4-AS1 is a potential marker in the diagnosis and treatment of thyroid cancer.

Author contributions

S.X., X.Y.Z., X.J.Y., J.W.W., H.Z., J.H.W., Q.Q.L., and R.Q.Y. made substantial contributions to conception and design, acquisition of data, analysis and interpretation of data, and draft of the manuscript. R.Q.Y. revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

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Ethical approval and consent to participate

All procedures were approved by the Ethics Committee of Shenzhen Bao'an Traditional Chinese Medicine Hospital, Guangzhou University of Chinese Medicine, and each of the research subjects signed an informed consent form.

Consent for publication

NA.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Conflict of interests

The authors report there are no competing interests to declare.

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NA.

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