European Review for Medical and Pharmacological Sciences 2020; 24: 12224-12231

CCAT2 contributes to progression and treatment resistance of thyroid carcinoma

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Abstract. – OBJECTIVE: The aim of this study is to uncover the correlations of the expression of colon cancer associated transcript 2 (CCAT2) in the clinical papillary thyroid carcinoma (PTC) and anaplastic thyroid carcinoma (ATC) specimens with the prognosis and chemoresistance of patients.

PATIENTS AND METHODS: The expression level of CCAT2 in the PTC and ATC specimens was determined using Real-Time quantitative Polymerase Chain Reaction (RT-qPCR), and the correlations of CCAT2 expression with the clinical features of patients were detected via χ2 test. Besides, survival analysis was conducted to verify the relation between CCAT2 expression and patients' survival. After knockdown or overexpression of CCAT2, the changes in the proliferation ability of human thyroid carcinoma cells were examined via Cell Counting kit-8 (CCK-8) assay, and the half maximal inhibitory concentration (IC50) values of doxorubicin and cisplatin were measured by methyl thiazolyl tetrazolium (MTT) assay.

RESULTS: According to the χ2-test results, the expression of CCAT2 was notably correlated with the capsular invasion and lymph node metastasis of PTC, and the capsular invasion, tumor size, and lymph node metastasis of ATC. It was discovered through the survival analysis that the expression of CCAT2 was notably associated with the poor prognosis of ATC patients. After knockdown of CCAT2, both the proliferation ability and the IC50 values of doxorubicin and cisplatin substantially declined in human thyroid carcinoma cells. The opposite conditions were found after CCAT2 was overexpressed in human thyroid carcinoma cells.

CONCLUSIONS: CCAT2 potentiates the proliferation ability and chemoresistance of cells, promotes the progression of thyroid carcinoma, and hinders the prognosis of ATC.

Key Words:
Anaplastic thyroid carcinoma, Papillary thyroid carcinoma, CCAT2.

Introduction

Thyroid carcinoma, one of the most common malignant tumors in the adult endocrine system, accounts for about 96% of the total endocrine system malignancies[1]. Meanwhile, the epidemiological statistics have shown that the morbidity rate of thyroid carcinoma is increasing rapidly in different age and sex groups all around the world over the past several decades, and that its increase in the morbidity rate is far higher than that of all other malignancies[1-3].

Clinically, thyroid carcinoma is classified into many pathological types, including papillary thyroid carcinoma (PTC), follicular thyroid carcinoma, medullary thyroid carcinoma, and anaplastic thyroid carcinoma (ATC), of which PTC is the most common and represents approximately 70-85% of all malignant thyroid tumors[4,5]. As a whole, PTC is now believed to be an indolent tumor characterized by favorable differentiation, slow growth, and low malignancy[6], but a certain proportion of patients suffers from highly malignant PTC, most of whom die within 5 years since the definite diagnosis[7,8]. In contrast with other pathological types of thyroid carcinoma, lung cancer, esophageal cancer and other common malignancies in clinic, ATC can be considered to be rare[9], and most (over half) deaths related to thyroid malignancies are caused by this disease with a clinical morbidity rate of only 1-5% at present[10,11].

Long non-coding ribonucleic acids (lncRNAs) are a class of over 200 nt-long RNAs that are not involved in protein coding, but participate in regulating gene expression at many levels[12]. LncRNAs are closely correlated with the development and progression of thyroid carcinoma and they regulate gene expression at the transcriptional, post-transcriptional and epigenetic levels,

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thereby exerting important regulatory effects on the proliferation, apoptosis, invasion and metastasis of thyroid carcinoma cells[13]. Colon cancer associated transcript 2 (CCAT2) is a novel lncRNA transcript, located on chromosome 8q24.21 with the single nucleotide polymorphism rs6983267. Its expression can be affected by heterogeneous rs6983267 genotypes, so that the risk allele G can produce more CCAT2 that is regarded as a biomarker for the poor prognosis and metastasis of tumors[14]. Currently, there have not yet been study reports on the correlation between CCAT2 and thyroid carcinoma. Therefore, the associations of CCAT2 expression with the clinical features and prognosis of thyroid carcinoma patients and its role in chemoresistance were analyzed in this study, so as to provide basic theoretical support for improving the efficacy and prognosis in thyroid carcinoma patients.

Patients and Methods

Patients

The thyroid carcinoma patients treated in The Ninth People's Hospital of Suzhou from June 2017 to June 2019 were enrolled as the subjects, and they underwent radical thyroid resection and had thyroid carcinoma proved by pathology. Among them, there were 60 cases each of PTC and ATC. Staging was conducted according to the Union for International Cancer Control (UICC) tumor node metastasis (TNM) staging classification criteria for thyroid carcinoma[15]. Inclusion criteria: patients with no severe diseases in other organs, those undergoing no post-operative radiotherapy and those with normal thyroid function before operation. Exclusion criteria: patients with distant metastasis or lung metastasis of tumors, those complicated with other malignancies, those with mental disease, those complicated with myocardial infarction, heart failure or other chronic diseases, those with abnormal thyroid function prior to operation, or those previously exposed to radioactive rays. The present investigation was approved by the Ethics Committee of The Ninth People's Hospital of Suzhou, and all patients signed the informed consent.

Follow-Up

All patients were followed up by outpatient re-examination and telephone until June 2019. The major observation endpoint was overall survival (OS) that refers to the duration from the definite diagnosis to the death of patients due to any cause. Additionally, there were no cases of loss to follow-up.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNAs were extracted from the thyroid carcinoma tissues according to the instructions of the TRIzol kit (Thermo Fisher Scientific, Waltham, MA, USA) and reversely transcribed into complementary deoxyribonucleic acids (cDNAs) using the reverse transcription kit (Beijing TransGen Biotechnology Co., Ltd., Beijing, China). The primers were provided by Shanghai Sangon Co., Ltd., with the primer sequences as below: CCAT2 F: 5'-CCCTGGTCAAATTGCTTAACCT-3', and R: 5'-TTATTCGTCCCTCTGTTTTATGGAT-3', GAPDH F: 5'-GCTCTCTGCTCCTCCTGTTC-3', and R: 5'-ACGACCAAATCCGTTGACTC-3'. Subsequently, RT-qPCR was performed using QuantiTect SYBR Green RT-PCR kit (Beijing Tiangen Biotech Co., Ltd., Beijing, China) and LightCycler® 96 Real-Time PCR System (Roche, Basel, Switzerland) (20 μL in total) consisting of 7.5 μL of SYBR Premix Ex Taq II (2×), 2.0 μL of cDNAs, 0.5 μL each of forward and reverse primers and 9.5 μL of ddH_2O. The RT-qPCR protocol was as follows: 95°C for 2 min, 95°C for 30 s, 59°C for 30 s and 72°C for 30 s for 30 cycles in total. At the end of reaction, the data of cycle threshold (Ct) of samples were obtained, and the relative messenger RNA (mRNA) expression level of CCAT2 was obtained by the $2^{-\Delta\Delta Ct}$ relative quantification method.

Cell Culture and Transfection

Human thyroid carcinoma cells were bought from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) in an incubator with 5% CO_2 at 37°C for 3-5 d, followed by sub-culture. Then, the passage 2-3 cells in the logarithm phase were harvested for subsequent researches. The cells in the logarithm phase were first seeded into 6-well plates, and upon reaching about 80% confluence, the cells were transfected using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) and further cultured for 24 h until the subsequent assays. Si-CCAT2 sense: 5'-AGGUGUAGCCAGAGUUAAUTT-3' and anti-sense: 5'-AUUAACUCUGGCUACACCUTT-3'.

Cell Counting Kit (CCK)-8 Assay

The cells in the logarithm phase were collected, and the cell suspension was seeded into 96-well plates at the adjusted concentration of 1×10^4 cells/mL and 100 μL/well, with 12 parallel wells set in each group. After culture for 24 h, the original medium was discarded, and 100 μL of fresh medium was added to each well that was then added with 10 μL of CCK-8 solution (Dojindo Molecular Technologies, Kumamoto, Japan) at 0, 1, 2 and 3 d. Following 4 h of culture, the absorbance of cells was measured with a microplate reader at a wavelength of 450 nm. Inhibitory rate of cell proliferation (%) = (absorbance in blank group – that in experiment group)/that in blank group ×100%.

Detection of Cell Sensitivity to Chemotherapeutic Drugs Via Methyl Thiazolyl Tetrazolium (MTT) Assay

The transfected cells were trypsinized and fully pipetted up and down into single-cell suspension. Then, 100 μL of single-cell suspension was evenly distributed into 96-well plates at 1×10^5 cells/mL and cultured in the CO_2 incubator overnight, with 6 replicates in each group. Subsequently, the cells were added with the clinical chemotherapeutic agents for thyroid carcinoma: cisplatin at 1 μg/mL or doxorubicin at 5 μg/mL, and cultured in the CO_2 incubator for another 48 h. Following addition with 20 μL of MTT reagent (Millipore, Billerica, MA, USA), the cells were further incubated in the incubator for 1 h. At the end of culture, the MTT-containing medium was carefully removed, and each well was added with 100 μL of dimethylsulfoxide (DMSO) to dissolve all purple crystals. Finally, the absorbance of cells at a wavelength of 490 nm using the microplate reader, based on which the cell growth curves were plotted and the half maximal inhibitory concentration (IC50) value of the chemotherapeutic drugs in cells.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA) was employed to analyze data. The normally distributed measurement data were presented as mean ± standard deviation (mean± SD) and compared between the two groups using *t*-test. Enumeration data were expressed as n (%) and analyzed by χ^2-test. The survival curves of thyroid carcinoma patients were drawn by the Kaplan-Meier method and GraphPad Prism 7 software (La Jolla, CA, USA). The differences in the 5-year OS among patients with different expression levels of CCAT2 were analyzed by log-rank test.

Results

Analysis of Correlations of CCAT2 Expression with Clinicopathological Features of PTC

The expression level of CCAT2 in the tumor tissue specimens collected from PTC patients *via* RT-qPCR, and with the median expression level as the boundary, the specimens were then assigned into high and low expression group. It was found through the χ^2-test that CCAT2 expression in PTC tissues was not correlated with the gender, age and tumor size of patients, but associated with the capsular invasion and lymph node metastasis of tumors. Moreover, the tumor tissues with capsular invasion and lymph node metastasis had a considerably higher expression level of CCAT2 than those without capsular invasion and lymph node metastasis (Table I). These above results suggested that CCAT2 can affect the invasion and metastasis of PTC.

Analysis of Correlations of CCAT2 Expression with Clinicopathological Features of ATC

First, the CCAT2 expression level in the tumor tissues collected from ATC patients was determined using RT-qPCR, and the tumor tissues were then divided into high and low expression group, with the median expression level as the boundary. Based on the χ^2-test results, the CCAT2 expression level in ATC tissues was not associated with the gender and age of patients, but correlated with the capsular invasion, size and lymph node metastasis of tumors. The expression of CCAT2 in tumor tissues with capsular invasion, a size of >1 cm and lymph node metastasis was notably higher than that in tumor tissues with no capsular invasion and lymph node metastasis (Table II). These findings imply that CCAT2 can influence the capsular invasion, tumor size, and lymph node metastasis of ATC.

Relationship Between CCAT2 and OS Rate of Thyroid Carcinoma Patients

To further elucidate the correlation between CCAT2 and patients' prognosis, the recruited 120 patients were followed up for up to 5 years. According to the analysis results of the survival curves, there was no correlation between CCAT2

Table I. Analysis of correlations of CCAT2 expression with clinicopathological features of PTC.

Variable	n	Low level (n=30)	High level (n=30)	χ^2	p
Sex					
Male	32	14	18	1.071	0.438
Female	28	16	12		
Age					
<60	27	10	17	3.300	0.119
≥60	33	20	13		
Capsular invasion					
No	31	21	10	8.076	0.009
Yes	29	9	20		
Tumor diameter (cm)					
≤1	34	21	13	4.344	0.067
>1	26	9	17		
Lymphatic metastasis					
No	26	18	8	6.787	0.018
Yes	34	12	22		

expression level with the OS rate of PTC patients [hazard ratio (HR) =2.520, p=0.1124] (Figure 1A). Besides, the OS rate of ATC patients in high expression group was remarkedly higher than that in low expression group (HR=7.128, p=0.0076) (Figure 1B). It can be inferred from these results that ATC patients with highly expressed CCAT2 have short survival and a poor prognosis.

CCAT2 Enhanced Proliferation Ability of Human Thyroid Carcinoma Cells

In vitro assays were conducted to explore the mechanism of action of CCAT2 in the development and progression of thyroid carcinoma. Based on the RT-qPCR results, the expression of CCAT2 substantially declined after CCAT2 was knocked down in human thyroid carcinoma cells (Figure 2A), and it was considerably raised after overexpression of CCAT2 (Figure 2B), suggesting that the transfection efficiency was eligible for the subsequent investigations. Besides, it was discovered through the CCK-8 assay that the proliferation ability declined in CCAT2 knockdown human thyroid carcinoma cells (Figure 2C), but the opposite conditions were detected after overexpression of CCAT2 (Figure 2D). The above results demonstrate that CCAT2 can enhance the proliferation ability of human thyroid carcinoma cells.

Effect of CCAT2 on Chemosensitivity of Human Thyroid Carcinoma Cells

To verify whether CCAT2 can affect the chemotherapy for thyroid carcinoma, the sensitivity of cells to chemotherapeutic drugs was determined

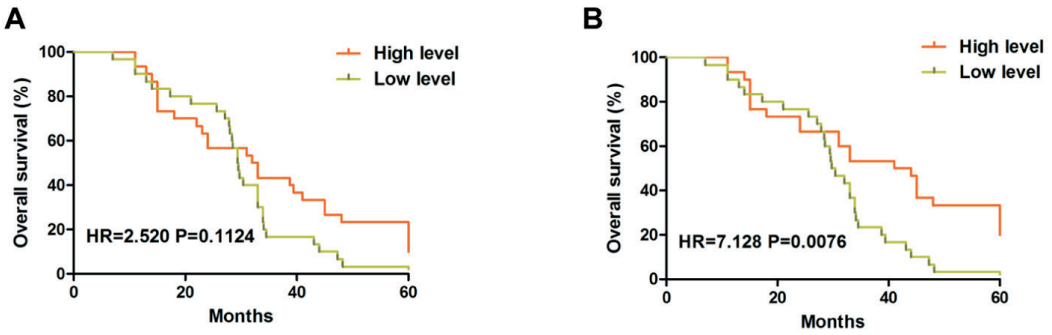

Figure 1. Relationship between CCAT2 and OS rate of thyroid carcinoma patients. **A,** The expression level of CCAT2 was not correlated with the OS rate of PTC patients (HR=2.520, p=0.1124). **B,** ATC patients with highly expressed CCAT2 had an obviously lower OS rate than those with lowly expressed CCAT2 (HR=7.128, p=0.0076).

Table II. Analysis of correlations of CCAT2 expression with clinicopathological features of ATC.

Variable	n	Low level (n=30)	High level (n=30)	χ^2	*p*
Sex					
Male	27	15	12	0.606	0.604
Female	33	15	18		
Age					
<60	25	9	16	3.360	0.115
≥60	35	21	14		
Capsular invasion					
No	33	21	12	5.455	0.037
Yes	27	9	18		
Tumor diameter (cm)					
≤1	29	19	10	5.406	0.038
>1	31	11	20		
Lymphatic metastasis					
No	28	20	8	9.463	0.004
Yes	32	10	22		

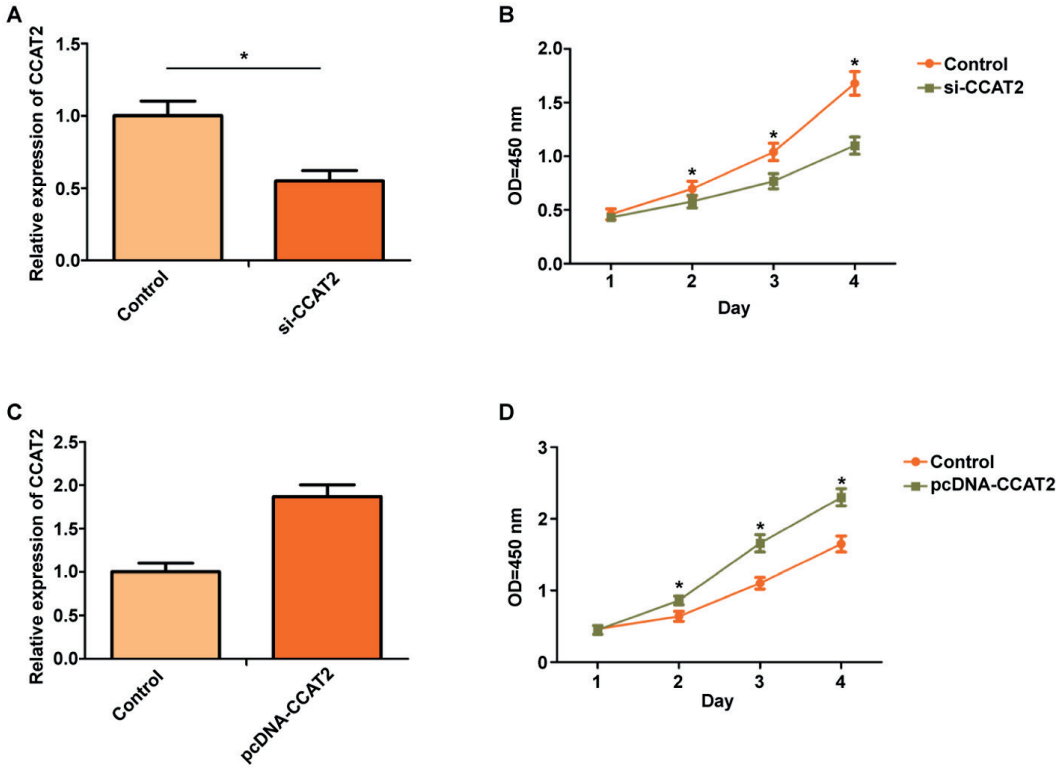

Figure 2. CCAT2 enhanced the proliferation ability of human thyroid carcinoma cells. **A,** The expression of CCAT2 considerably declined in human thyroid carcinoma cells with the knockdown of CCAT2. **B,** The expression of CCAT2 was remarkably up-regulated in human thyroid carcinoma cells after overexpression of CCAT2. **C,** The CCK-8 assay results showed that the proliferation ability declined in CCAT2 knock-down human thyroid carcinoma cells. **D,** Based on the CCK-8 assay results, the proliferation ability of human thyroid carcinoma cells was enhanced after overexpression of CCAT2.

via MTT assay. According to the results, the IC50 value of cisplatin was remarkably decreased in the cells with knockdown of CCAT2 (Figure 3A), but it markedly rose after overexpression of CCAT2 (Figure 3B). Moreover, after treatment with doxorubicin, the IC50 value of doxorubicin

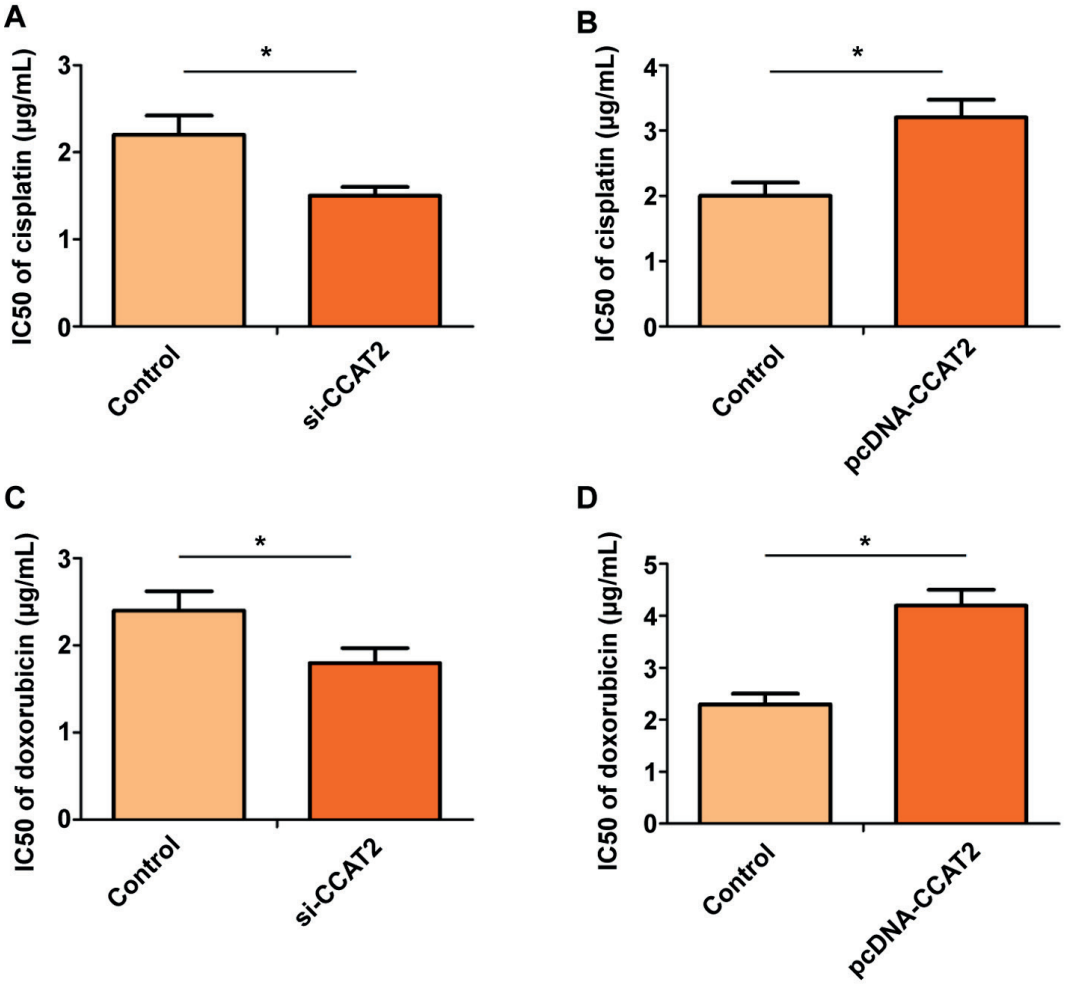

Figure 3. Effect of CCAT2 on chemosensitivity of human thyroid carcinoma cells. **A,** The IC50 value of cisplatin was notably decreased in the CCAT2 knock-down cells. **B,** The IC50 value of cisplatin was substantially raised in CCAT2 overexpression cells. **C,** After knockdown of CCAT2, the IC50 value of doxorubicin considerably declined in the cells. **D,** After overexpression of CCAT2, the IC50 value of doxorubicin dramatically rose in the cells.

was considerably decreased in the CCAT2 knock-down cells (Figure 3C), but prominently rose in the CCAT2 overexpression cells (Figure 3D).

Discussion

Thyroid carcinoma is not only a common thyroid malignancy, but also a common malignant tumor of the endocrine system. Statistics have shown that the thyroid carcinoma has the fastest increase in the morbidity rate among all malignant solid tumors, severely affecting human health[16,17]. However, the specific pathogenesis of thyroid carcinoma has not yet been fully clarified. It has now been believed that 90% of human malignancies are associated with environmental factors, and that environmental changes induce the alterations in some pathogenic genes, thereby promoting the development of diseases[3].

LncRNAs are far less conservative than coding RNAs, but lncRNA molecules have conserved local segments inside and their expression exhibits spatiotemporal specificity. These phenomena indicate that lncRNAs have important physiological and biochemical functions in tumors[18-20]. Moran et al[21] demonstrated that lncRNAs are implicated in regulating many biological processes, such as X-chromosome silencing and transcriptional interference, as well as chromatin modification, transcriptional activation, and nuclear transport.

LncRNAs may exert an important function in multiple cancers[22]. Correspondingly, BRAF-ac-

tivated non-protein coding RNA (BANCR) dramatically rises, while PTCSC3 and NAMA remarkably decline in thyroid carcinoma tissues compared with those in normal thyroid tissues. Moreover, BANCR is closely correlated with the modulation of thyrotropin receptor expression[23]. Additionally, downregulating lncRNA ANRIL can suppress the proliferation and migration of PTC cells[24]. CCAT2, a lncRNA sequence firstly discovered in colon cancer, can be overexpressed to promote the growth and metastasis of tumors in colon cancer[25]. CCAT2 may play an important regulatory role in multiple cancers. CCAT2 is highly expressed in cervical cancer and regulates the growth and apoptosis of cervical cancer cells[26], and it is also highly expressed in liver cancer and modulates the proliferation, apoptosis and migration of liver cancer cells[27].

The results of this study showed that high expression of CCAT2 contributed to the capsular invasion and lymph node metastasis of PTC and ATC, and it increased the tumor size of ATC and worsened the prognosis of ATC patients. The *in vitro* cell assay results showed that CCAT2 enhanced the proliferation ability of human thyroid carcinoma cells, thereby facilitating the progression of thyroid carcinoma. Cisplatin, a platinum-containing chemotherapeutic drug, is one of the commonly used chemotherapeutic drugs for the treatment of advanced or metastatic undifferentiated thyroid cancer[28]. Lamb et al[29] reported that antibiotics can affect tumor growth by targeting mitochondria and eliminating tumor stem cells. Macrolide antibiotics, such as azithromycin, blocks autophagy and thus sensitizes apoptosis of tumor cells[30,31]. Our results identified that overexpression of CCAT2 raised the IC50 values of doxorubicin and cisplatin in human thyroid carcinoma cells, but their IC50 values were substantially lowered by the knockdown of CCAT2, indicating that CCAT2 can promote the chemoresistance of human thyroid carcinoma cells through the MTT assay.

Therefore, CCAT2 exerts a crucial role in the progression of thyroid carcinoma and can affect the proliferation and chemoresistance of thyroid carcinoma cells. In this study, we explored biomarkers responsible for tumor cell proliferation and chemotherapy resistance of thyroid cancer. Cancer biomarkers can be non-invasively detected and monitored, which present a promising clinical application with high sensitivity and specificity. Moreover, they also can be utilized for developing therapeutic targets for the diagnosis and treatment of thyroid cancer.

Conclusions

Briefly, the above data demonstrated that CCAT2 promotes the proliferation of human thyroid carcinoma cells, thereby driving the progression and chemoresistance of thyroid carcinoma.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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