

## Original Article

# Long noncoding RNA CCAT2 promotes proliferation and metastasis in non-small cell lung cancer through the Wnt pathway

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**Abstract:** Colon cancer associated transcript 2 (CCAT2), a long non-coding RNA (lncRNA), was shown to be associated with colon, ovarian and prostate cancer. Recent studies showed CCAT2 was highly expressed in various tumors, including non-small cell lung cancer (NSCLC) and probably being an independent prognostic factor of this disease. However, the physiological and biochemical mechanisms for CCAT2 with NSCLC were still unknown. In this study, we performed the analysis focused on the expression, biological functions and mechanism of CCAT2 in NSCLC and found that CCAT2 was significantly up-regulated in NSCLC tissues compared with corresponding non-cancerous tissues. Knockout of CCAT2 in NSCLC cell lines using lentivirus-mediated RNA interference significantly inhibited the proliferation and metastasis of the NSCLC cells. Most importantly, Wnt/ $\beta$ -catenin pathway was found to be inactivated in the NSCLC cell lines after CCAT2 knockout experiment. These results indicated that CCAT2 maybe serve as an oncogenic lncRNA that promoted proliferation and metastasis of NSCLC and activated the Wnt/ $\beta$ -catenin pathway.

**Keywords:** CCAT2, lncRNA, NSCLC, Wnt/ $\beta$ -catenin pathway

## Introduction

Lung cancer is a common malignant tumor for human kind, and is also the most common cause of death worldwide at present [1, 2]. Based on the differentiation and morphological characteristics, lung cancer can be divided into small cell lung cancer (SCLC) and non small cell lung cancer (NSCLC), and NSCLC accounted for nearly 85% all of the lung cancer [3-5]. Metastatic spread of tumor cells is the main cause of cancer death. The process of cancer metastasis consisted of linked sequential steps, including invasion, detachment, intravasation, circulation, adhesion, extravasation and growth in distant organs [6-8]. Currently, there were no reliable markers available to accurately predict metastasis in early-stage NSCLC patients. Therefore, the most important strategy for NSCLC is to find an effective marker of early diagnosis and prognosis, and provide a theoretical basis for the invasion and metastasis of cancer cells.

Long non-coding RNAs (lncRNAs) can be used as gene regulators of controlling protein-coding and non-coding genes [9]. The altered expression of lncRNAs was frequently observed in human cancers [10]. Studies have shown that the primary structure, secondary structure of lncRNAs and changes in their binding proteins were associated with metastasis, invasion, and patient survival [10, 11]. These findings suggested that the role of lncRNAs in the process of cancer development. lncRNA is generally refers to RNA longer than 200 nt in the nucleus or cytoplasm, doesn't participate in protein coding function, and in the form of RNA regulation of gene expression in multiple levels [12] CSIR-Institute of Genomics and Integrative Biology (CSIR-IGIB). Their abilities are to regulate essential pathways for tumor initiation and progression, and promot them as valuable biomarkers and therapeutic targets. In 2013, Ling et al [13] first discovered colon cancer associated transcript 2 (CCAT2), a novel non-coding RNA located at 8q24 of human being. They

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**Table 1.** Primers used in this study

Genes	Primers	Sequences (5'-3')
CCAT2	P1	CCCTGGTCAAATTGCTTAACCT
	P2	TTATTCGTCCTCTGTTTTATGGAT
GAPDH	P1	AGAAGGCTGGGGCTCATTG
	P2	AGGGGCCATCCACAGTCTTC

**Table 2.** The clinical and pathological information of the patients

Groups		Numbers
Sex	Male	17
	Female	36
Age	Mean	60.8±5.2
Tumor differentiation	Well	11
	Moderate	26
	Poor	16
Tumor invasion	T1	6
	T2	14
	T3	17
	T4	16
Lymph node metastasis	Absence	19
	Presence	31
	No	3
Distant metastasis	Absence	48
	Presence	5
TNM stage	I	6
	II	11
	III	20
	IV	16

found that CCAT2 was highly over-expressed in microsatellite-stable colorectal cancer and promotes tumor growth, metastasis, and chromosomal instability. Recent studies [14-16] showed that CCAT2 was highly expressed in various tumors, including NSCLC. However, the physiological and biochemical mechanisms for CCAT2 with NSCLC were still unknown. In this study, we performed the analysis on the expression, biological functions and mechanism of CCAT2 in NSCLC.

### Materials and methods

#### *Patient samples and cell lines*

NSCLC and paired corresponding normal non-cancerous tissues were obtained from 53 patients, underwent surgery resection in the Cancer Hospital of China Medical University

between 2012 and 2016. The patients were recruited in our study for no radio or chemotherapy before the surgery. The clinical information of the patients was collected as well. All the specimens were frozen and stored at -70°C. This study was approved by the research ethic committee of our hospital, China. All the written informed consents were got from patients for biological research.

Three NSCLC cell lines (PC9, SPC-A1 and H1299) and one normal tissue (16HBE) were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in RPMI-1640 medium (GIBCO, USA) supplemented with 10% fetal bovine serum (10% FBS), and maintained in the humidified incubator at 37°C with 5% CO<sub>2</sub>.

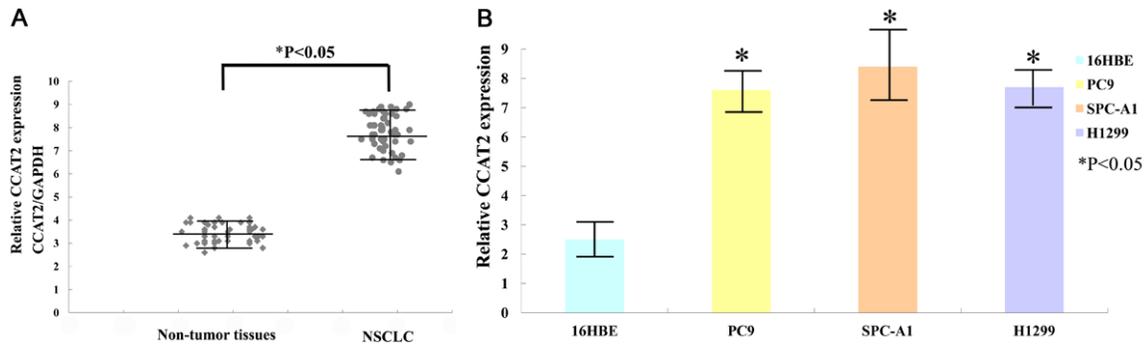
#### *RNA extraction and qRT-PCR assays*

Total RNA was isolated from tissues and cell cultures with Trizol reagent (TaKaRa, Japan) according to the manufacturers' instructions. The cDNA was reverse transcribed from a total of 200 ng RNA using the PrimeScript RT reagent Kit (TaKaRa, Japan) and amplified by quantitative real-time PCR by SYBR Green Kit (TaKaRa, Japan) on ABI 7500 (USA). GAPDH was used as the internal reference, and the relative expression of CCAT2 was normalized to GAPDH. Primers for CCAT2 and GAPDH were shown in **Table 1**.

#### *Interfering RNA construction*

The small interfering RNA (siRNA) against CCAT2 and the negative control (si-NC) were used and synthesized by GenePharma (Shanghai, China). The following short hairpin RNA was used to target CCAT2: sense: 5'-UUAACCUC-UUCCUAUCUCATT-3'; antisense: 5'-UGAGAU-GGAAGAGGUUAATT-3'. The negative control sequence was 5'-UUCUCCGAACGUGUCACGUTT-3'. The cells were cultured on six-well plates in the density of 5×10<sup>5</sup>, and the cells were transfected with siRNA and si-NC by using Lipofectamine 2000 (Invitrogen, USA) at 100 nM concentration. The interfering results were determined by qRT-PCR after transfection 48 hours, and the silencing efficacy of siRNA were used for more than 85% to further investigation.

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**Figure 1.** Comparison of CCAT2 in NSCLC tissues and cell lines. A. Relative CCAT2 expression in NSCLC and non-tumor tissues. B. Relative CCAT2 in NSCLC cell lines. \* $P < 0.05$ .

### Cell proliferation

Cell proliferation was performed by using Cell Counting Kit (CCK-8, Dojindo) based on the manufacturer's instruction. 96-well plates were used for three groups (control, siRNA and si-NC), 100  $\mu$ l cells were inoculated into the plates at 1000 cells/well, and cultured at 37°C. The densities of the cells were measured at 450 nm, and the survival rate was expressed as the absorbance relative to the control group.

For colony forming experiments, the cells transfected with siRNAs and si-NC were inoculated into six well plates, maintained in RPMI-1640 medium contained 10% FBS for two weeks. Colonies were fixed with methanol 12 days later and stained with 0.1% crystal violet (Sigma, USA). The colonies were photographed and counted manually.

### Cell cycle and apoptosis

Cells were harvested after transfected by trypsin and centrifugation. Then, washed with cold PBS, and fixed with 70% ethanol at 4°C. The cells were stained with propidium iodide at 4°C for 30 min. The results were analyzed with flow cytometer FACScalibur (BD, USA).

For the apoptosis analysis, the trypsinized and washed cells were stained with FITC-Annexin V and propidium iodide, and analyzed by FACScalibur and Flowjo software (BD, USA). Cells were discriminated for viable cells, dead cells, early apoptosis cells and apoptosis cells.

### Western blotting

Cells cultures were lysed with protein extraction reagent RIPA (Beyotime, China). The pro-

teins were separated by SDS-PAGE and transferred to PVDF membranes. The blots were probed with primary antibodies at 4°C: anti- $\beta$ -catenin, anti-CCND1, and anti-c-myc. The GAPDH was used as the internal control.

### Statistical analysis

SPSS Statistics for Windows, Version 17.0. (SPSS Inc., Chicago, USA) was used for statistical analysis of the data. Data were shown as mean  $\pm$  standard deviation (STD). Statistical significance was tested by Student's t-test, Chi-square test and one way ANOVA if appropriate. Two tailed  $P < 0.05$  was considered to be statistically significant.

## Results

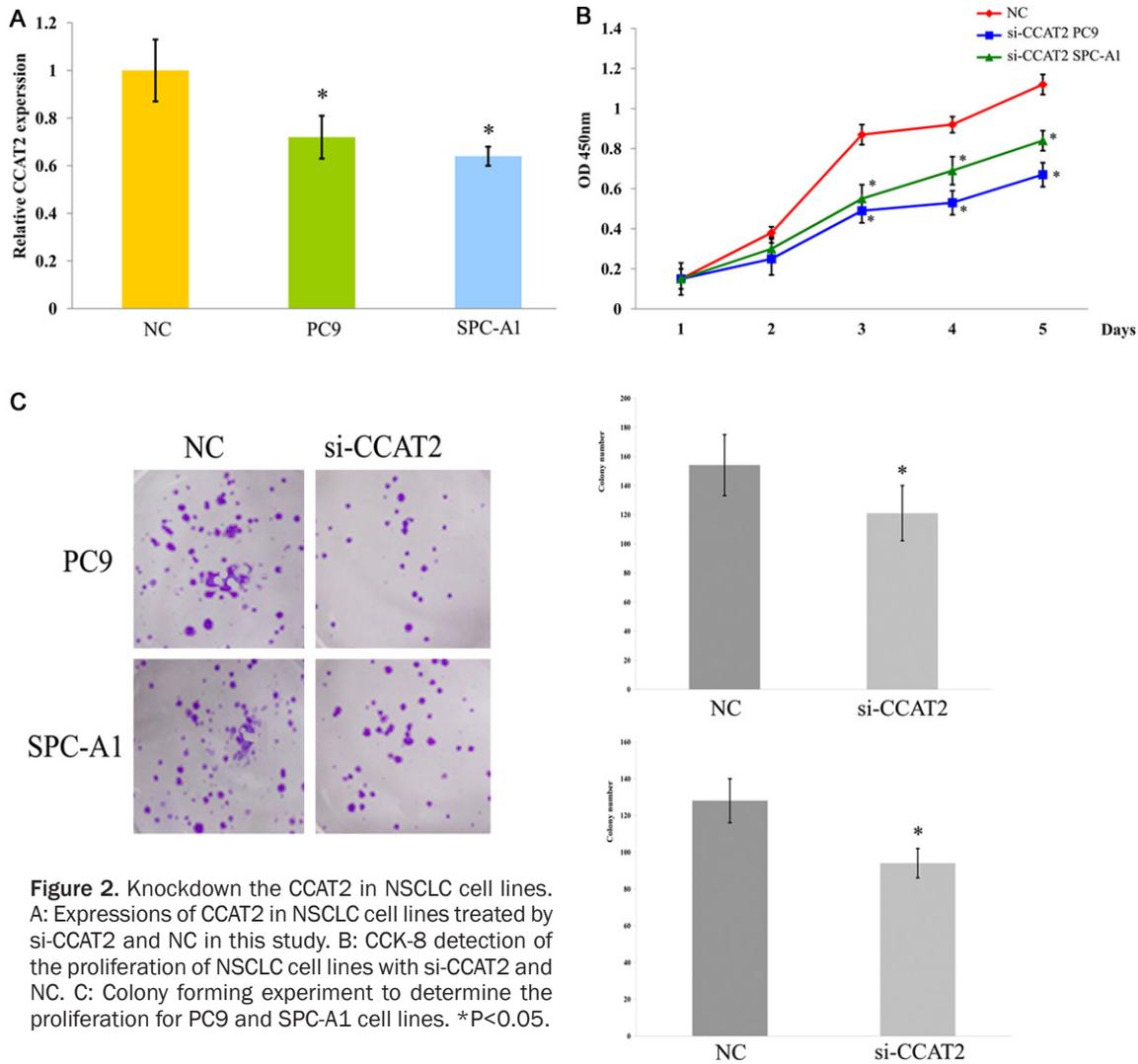
### CCAT2 were up-regulated in NSCLC tissues and cell lines

The qRT-PCR was performed to evaluate the expression of CCAT2 in 53 NSCLC and corresponding non-tumor tissues. The clinical and pathological information of the patients were shown in **Table 2**. The level of CCAT2 was increased significantly in NSCLC tissues compared with the non-tumor tissues (**Figure 1A**). The cell lines comparison results also showed highly expressed CCAT2 to the NSCLC cells (**Figure 1B**).

### Knockdown of CCAT2 in NSCLC cell lines

The CCAT2 was knockdown in NSCLC cell lines, accessed the role of CCAT2 in NSCLC growth. Two cell lines were used, PC9 and SPC-A1. The decreased CCAT2 expression levels were selected for more than 85%. The CCAT2

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**Figure 2.** Knockdown the CCAT2 in NSCLC cell lines. A: Expressions of CCAT2 in NSCLC cell lines treated by si-CCAT2 and NC in this study. B: CCK-8 detection of the proliferation of NSCLC cell lines with si-CCAT2 and NC. C: Colony forming experiment to determine the proliferation for PC9 and SPC-A1 cell lines. \*P<0.05.

expressions were statistically significant decreased in all the cell lines (**Figure 2A**). Cell proliferation results detected by CCK-8 revealed that NSCLC cells growth were inhibited both in PC9 and SPC-A1 compared with negative control (**Figure 2B**), and the colony forming experiment also showed reduced phenomenon by CCAT2 silenced in PC9 and SPC-A1 cell lines (**Figure 2C**).

### *The cell cycle progression and apoptosis of the NSCLC cell lines*

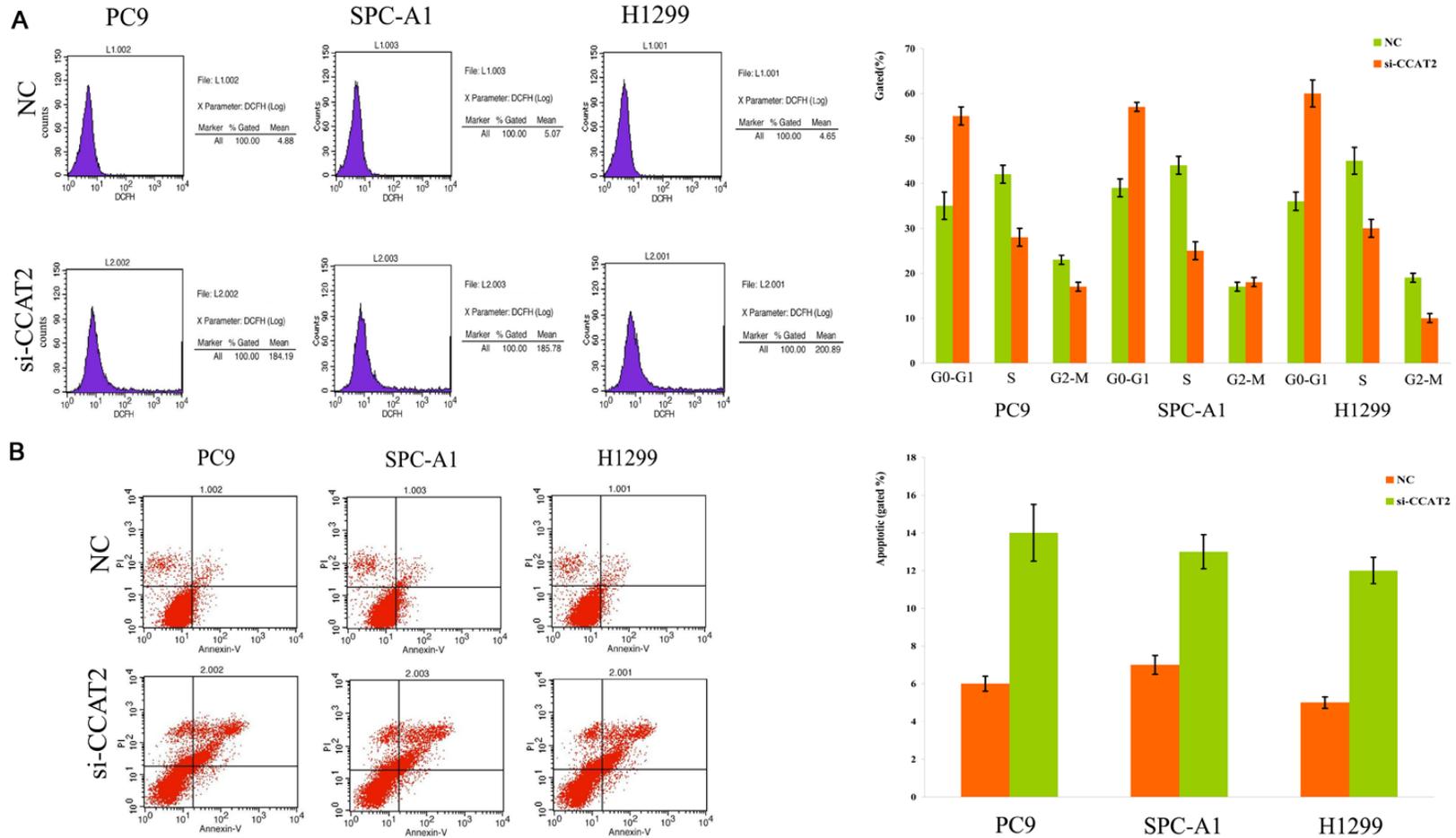
We performed the cell-cycle arrest and apoptosis test for knockdown CCAT2 in NSCLC cell lines. The results showed that si-CCAT2 of PC9, SPC-A1 and H1299 had an obvious cell-cycle arrest at the G1-G0 phase and had a decreased at G2-S phase (**Figure 3A**). The flow-cytometry and TUNEL staining analysis was also per-

formed, and the results showed that all the NSCLC cell lines transfected with si-CCAT2 increased the apoptosis proportion compared with the control groups (**Figure 3B**). These data suggested that si-CCAT2 could promote the proliferation of NSCLC cell lines.

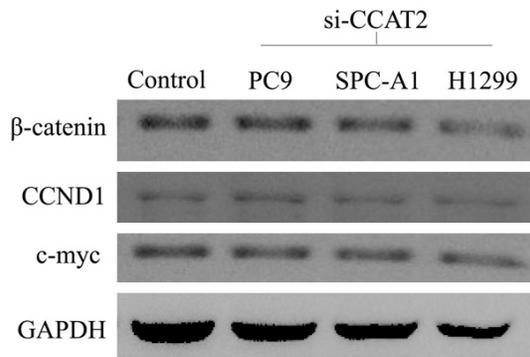
### *Si-CCAT2 on the signal regulated Wnt/ $\beta$ -catenin pathway*

CCAT2 regulated NSCLC cells' proliferation and metastasis mechanisms were determined by western blot, and the results showed that the  $\beta$ -catenin, CCND1, and c-myc were significantly reduced for knockdown of CCAT2 compared with the control (**Figure 4**). These data suggested that Wnt/ $\beta$ -catenin pathway might involve in the CCAT2 mediated proliferation and metastasis of NSCLC cell lines.

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**Figure 3.** Effects of knockdown the CCAT2 gene on NSCLC cell lines for cell cycle and apoptosis analysis. A: The percentage of cell in G0-G1, S and G2-M phase. B: The apoptosis analysis by flow cytometry.



**Figure 4.** Western blot results of the Wnt/ $\beta$ -catenin pathway related proteins in NSCLC cell lines.

**Discussion**

NSCLC is any type of epithelial lung cancer other than small cell lung carcinoma (SCLC) [17, 18]. NSCLC accounted for about 85% of all lung cancers. As a class, NSCLCs were relatively insensitive to chemotherapy, compared to small cell carcinoma. When possible, they were primarily treated by surgical resection with curative intent, although chemotherapy was increasingly being used both pre-operatively and post-operatively [19-22]. Therefore, the most important strategy for NSCLC was to find an effective marker of early diagnosis and prognosis of cancer cells. With the rapid development of high-throughput gene transcription methods, a large number of evidences showed that at least 90% of the mammalian genome was active transcription, but only less than 2% of the genome sequence was capable of encoding proteins. Genome transcription contained a lot of non-coding RNAs, including small RNAs (miRNA) and long chain non-coding RNAs (lncRNA) [9, 23]. It was generally believed that lncRNA played an important role in cancer development, such as tumor formation, invasion and metastasis. lncRNA is a RNA molecule that is more than 200 nt in length and is not encoded protein. At present, lncRNA has become a hot research topic, which shows that the occurrence and development of a variety of tumors are closely related with lncRNA, and plays an important role for regulation effects, such as the homeobox gene antisense gene RNA (HOTAIR) [24], growth arrest specific transcript 5 (GAS5) [25], lung cancer metastasis related transcript 1 (MALAT1) [26].

Ling et al [13] first discovered CCAT2, a novel non-coding RNA located at 8q24, in 2013. After that, several studies showed that CCAT2 had close relationships with various tumors. For examples, Redis et al [16] showed that CCAT2 represented a valuable predictive marker of clinical outcomes (shorter metastases-free survival and overall survival) for a specific subgroup of breast cancer patients. Qiu et al [27] reported that CCAT2 was a lung adenocarcinoma-specific lncRNA and promoted invasion of non-small cell lung cancer, and CCAT2 could be used as a potential biomarker for lymph node metastasis. Wang et al [28] analyzed that CCAT2 correlated with smoking in esophageal squamous cell carcinoma, and that CCAT2 showed higher diagnostic performance than conventional serum biomarkers. In addition, Wang et al [29] reported that upregulation of CCAT2 was correlated with gastric cancer progression, and CCAT2 might be a potential molecular biomarker for predicting the prognosis of patients. These studies suggested that CCAT2 might exert its biological function as an oncogene in several kinds of cancers; however, the exact function of CCAT2 in NSCLC was still uncertain.

In this study, we performed a study in terms of the expression, biological functions and mechanism of CCAT2 in NSCLC and found that CCAT2 was significantly up-regulated in NSCLC tissues. Knockout of CCAT2 in NSCLC cell lines significantly inhibited the proliferation and metastasis of the NSCLC cells. Most importantly, Wnt/ $\beta$ -catenin pathway was found to be inactivated in the NSCLC cell lines after CCAT2 knockout experiment. These results indicated that CCAT2 maybe serve as an oncogenic lncRNA that promoted proliferation and metastasis of NSCLC and activated the Wnt/ $\beta$ -catenin pathway.

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**Disclosure of conflict of interest**

None.

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