

Long non-coding RNA CCAT2 promotes the breast cancer growth and metastasis by regulating TGF- β signaling pathway

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Abstract. – **OBJECTIVE:** Long non-coding RNA (LncRNA) CCAT2 plays an important role in tumorigenesis, tumor growth and metastasis. In this study, we reported that long noncoding RNA CCAT2 (LncRNA CCAT2) could regulate TGF- β signaling pathway in breast cancer.

PATIENTS AND METHODS: The relative mRNA expression level of CCAT2 in adjacent non-cancerous and breast cancer tissues without or with metastasis were analyzed by quantitative Real-time polymerase chain reaction (qRT-PCR). The mRNA expression levels of CCAT2 in breast cancer cell lines were analyzed by qRT-PCR. Proliferation, invasion and migration of breast cancer cells were detected after infected with si-NC or si-CCAT2. Flow cytometry analysis was used to detect the cell cycle distribution and apoptosis rate in breast cancer cells after infected with si-NC or si-CCAT2. The relative protein expression level of TGF- β , Smad2 and α -SMA in breast cancer cells after infected with si-NC or si-CCAT2 were analyzed by Western blot.

RESULTS: The relative mRNA expression level of CCAT2 in breast cancer tissues was significantly increased compared with adjacent non-cancerous tissues. The expression level of CCAT2 in breast cancer without metastasis was decreased compared with breast cancer metastasis. Meanwhile, down-regulation of CCAT2 inhibited the proliferation, invasion and migration in breast cancer cells. Furthermore, down-regulation of CCAT2 caused breast cancer cells cycle arrested in G0/G1 phase and promoted cell apoptosis. Down-regulation of CCAT2 significantly down-regulated the protein expression levels of TGF- β , Smad2 and α -SMA in breast cancer cells.

CONCLUSIONS: CCAT2 was highly expressed in breast cancer. Down-regulation of CCAT2 in-

hibited the proliferation, invasion and migration and promoted cell apoptosis in breast cancer cells by regulating TGF- β signaling pathway.

Key Words:

LncRNA CCAT2, Breast cancer, Metastasis, Proliferation, Invasion, Migration, Apoptosis, TGF- β .

Introduction

Breast cancer is the most common primary malignant tumor in women^{1,2}. The past few decades have witnessed profound advances in diagnosis and treatment of breast cancer³. Despite extensive efforts including chemotherapy, surgery, and sometimes radiotherapy, all together improved the diagnosis and treatment of breast cancer, but limited progress has been made^{4,5}. A major problem is a poor prognosis for metastatic relapse or recurrence⁶. Many studies⁷⁻⁹ have identified tumor suppressor genes and carcinogenes, which are believed to account for the growth of breast cancer. The detailed mechanisms of its tumorigenesis remain largely unknown. Therefore, it is in great need to understand the underlying mechanisms of breast cancer progression for the treatment of breast cancer. Long non-coding RNAs (LncRNAs) are a class of RNAs that have more than 200 nucleotides and have the ability to code proteins in animals and plants¹⁰⁻¹³. Accumulating evidence has shown they are important for specific binding and meaningful secondary structures

involved in multiple gene regulatory networks¹⁴⁻¹⁶. Several studies¹⁷⁻²⁰ have reported that lncRNAs with biological functions were associated with the proliferation, metastasis, invasion, migration and apoptosis in human cancer cells. Various lncRNAs have been demonstrated to play an important role in tumorigenesis including breast cancer²¹⁻²³. However, the detailed mechanism of lncRNAs is still awaiting for further elucidation.

lncRNA CCAT2 located in the highly conserved 8q24 region is discovered in many tumor formations^{24,25}. Studies showed CCAT2 was up-regulated in human gastric cancer, colorectal cancer. It promoted tumor growth and metastasis, and worked as a tumor promotor via long-range modulation of the expression of cell cycle-related genes^{26,27}. Lan et al²⁸ reported overexpression of CCAT2 promoted gastric cancer cell migration and invasion by regulating the MMPs and key EMT markers. However, the underlying mechanisms of CCAT2 expression in breast cancer and the mechanism remain to be uncovered.

In this investigation, the relative mRNA expression level of CCAT2 in adjacent non-cancerous and breast cancer tissues without or with metastasis were measured. The role of CCAT2 was elucidated in breast cancer. Breast cancer cells proliferation, invasion, migration, cell cycle distribution and apoptosis rate were detected after infected with si-NC or si-CCAT2. Our study showed down-regulation of CCAT2 inhibited the proliferation, invasion and migration and promoted cell apoptosis in breast cancer cells by regulating TGF- β signaling pathway.

Patients and Methods

Human Tissues

This research was approved by the Ethics Committee of the Ethics Committee of Chongqing Cancer Institute/Hospital. The patients or their legal guardian provided written informed consents to the surgical procedures and gave permission to use resected tissue specimens for research purposes. A total of 60 patients who were diagnosed with breast cancer were collected from Chongqing Cancer Institute/Hospital from 2015 to 2016. The patients with preoperative history of radiotherapy, chemotherapy, and positive surgical margins were excluded in our study. 36 breast cancer patients were involved with lymph node metastasis and 24 breast cancer patients without lymph node metastasis.

Tumor tissues and matched adjacent non-cancerous tissue (healthy controls) samples from patients were obtained and frozen by liquid nitrogen, immediately. Then the samples were stored at -80°C for later use.

Cell Culture and Transfection

The normal human breast cell line HCC1937 and breast cancer cell lines LCC9, MDA-MB_231 and MCF-7 were purchased from a cell bank at the Chinese Academy of Sciences and cultured in RPMI-1640 medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and antibiotics. All the cells were maintained under the recommended culture conditions and incubated at 37°C in a humidified environment with 5% CO₂.

Small interfering RNA against CCAT2 (si-CCAT2) and its negative control (si-NC) were synthesized by RiBo Biotech (Guangzhou RiBo Biotech). Breast cancer cells at a density of 1×10⁵ cells/well were seeded in each cell of a 24-well microplate, grown for 24 h to reach 30-50% confluence, and then transfected with si-CCAT2 or si-NC using Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The down-expression efficacy was confirmed by qRT-PCR and Western blotting analysis.

Real-time qPCR

Total RNA was severally extracted from tumor tissues and healthy controls using a Trizol kit (Invitrogen, Carlsbad, CA, USA). cDNA was subsequently synthesized from total RNA using an Omniscript RT kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RT-PCR reaction was conducted on the Mastercycler ep realplex (Eppendorf 2S, Hamburg, Germany). A 25- μ l-reaction mixture contained 1 μ l of cDNA from samples, 12.5 μ l of 2 X Fast EvaGreen™ qPCR Master Mix, 1- μ l primers (10 mM), and 10.5 μ l of RNase/DNase-free water. The Ct value was defined as the cycle number at which the fluorescence intensity reached a certain threshold where amplification of each target gene was within the linear region of the reaction amplification curves. Glyceraldheyde 3-phosphate dehydrogenase (GAPDH) was taken as internal controls. Relative mRNA expression of CCAT2 was calculated using the ²- $\Delta\Delta$ Ct method. The sequences of the primers for CCAT2 and GAPDH were as follows: CCAT2:

forward-5'-CCACATCGCTCAGACACCAT-3'; reverse-5'-ACCAGGCGCCCAATACG-3'. GAPDH: forward-5'-TGTTCCCTACCCCAATGTGTCC-3'; reverse-5'-GGAGTTGCTGTTGAA-GTCGCAG-3'. Each sample was in triplicate.

Cell Proliferation Assay

Cell proliferation was assessed by the CCK-8 assay. Briefly, LCC9 and MCF-7 cells were cultured in 96-well plates (3000 cells/well) and transfected with si-CCAT2 or si-NC for 48 h. Afterwards, 10 μ L CCK-8 solution (Dojindo Laboratories, Shanghai, China) was added to each well and then cells were incubated for additional 10 mins before proceeding to detection of absorbance at 405 nm. Air bubbles were strictly avoided during the process. Each sample was in triplicate.

Cell Invasion and Migration Assays

3×10^5 cells/ml of LCC2 and MCF-7 cells were respectively prepared after being transfected with si-CCAT2 or si-NC for 48 h. The cell migration and invasion capacity were determined using Transwell assay (Corning, Corning, NY, USA). Transfected cells were resuspended in serum-free medium. Then, 200 μ l cell suspensions were seeded into the upper chamber with a porous membrane coated with (for the Transwell invasion assay) or without (for the migration assay) Matrigel (BD Biosciences, San Diego, CA, USA). Complete medium was added to the bottom wells of the chambers. After migrating for 24 h or to invade for 48 h, the numbers of migrated and invasion cells were calculated by counting five different views under the microscope. Each sample was in triplicate.

Cell Cycle Analysis and Apoptosis Analysis

Each group of LCC9 and MCF-7 cells was seeded into six-well plates at a concentration of 3×10^5 cells/well after being transfected with si-CCAT2 or si-NC for 48 h. Afterwards, cells were collected by low-speed centrifugation (1000 rpm, 5 min) at 4°C and cell pellets were re-suspended in 1 ml of phosphate buffered saline (PBS) solution, fixed with 75% of ice-cold ethanol and stored at -20°C for two days. Prior to flow cytometry (FCM) analysis, cells were lysed, centrifuged and re-suspended in propidium iodide (PI) (Sigma-Aldrich, St. Louis, MO, USA) staining buffer containing 50 μ l/ml of PI and 250 μ l/ml of RNase A. Finally, the cell mixture was incubated at 4°C for 30 min in the dark

environment to detect cell cycle and stained with 5 μ L of annexin V-FITC to detect apoptosis by fluorescence activated cell sorting (FACS) technique (Beckman, Munich, Germany). Each sample was in triplicate.

Western Blot Assay

Breast cancer cells were harvested and lysed in lysis buffer. The mixture was centrifugation at 14000 g and 4°C for 30 min and a BCA assay was used to detect the concentration of protein in the supernatants using a Varioskan multimode microplate spectrophotometer (Thermo Scientific, Waltham, MA, USA). Proteins extracts were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transferred by semi-dry blotting to nitrocellulose membranes. The nitrocellulose membranes were blocked with 10% no-fat milk in phosphate buffered saline (PBS) at 37°C for 1 h and incubated with specific antibodies against indicated proteins overnight at 4°C. After washing three times for 10 min each in phosphate buffered saline tween (PBST), membranes were incubated with secondary antibody for 1 h at 37°C. Detection was performed using the Odyssey Infrared Imaging System (LI-COR Inc., Lincoln, NE, USA). Each sample was in triplicate.

Statistical Analysis

All data in the experiments were expressed as mean \pm SEM. Statistical analyses were performed by Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Paired and unpaired two-tail Student's *t*-test was applied to test the results. *p* < 0.05 was considered statistically significant.

Results

The Relative Expression Level of CCAT2 in Adjacent Non-cancerous and Breast Cancer Tissues without or with Metastasis

The relative mRNA expression level of CCAT2 in adjacent non-cancerous and breast cancer tissues without or with metastasis was analyzed by qRT-PCR. The relative mRNA expression level of CCAT2 in breast cancer tissues was significantly increased compared with adjacent non-cancerous tissues (Figure 1A) and the expression level of CCAT2 in breast cancer without metastasis was decreased compared with breast cancer metastasis (Figure 1B).

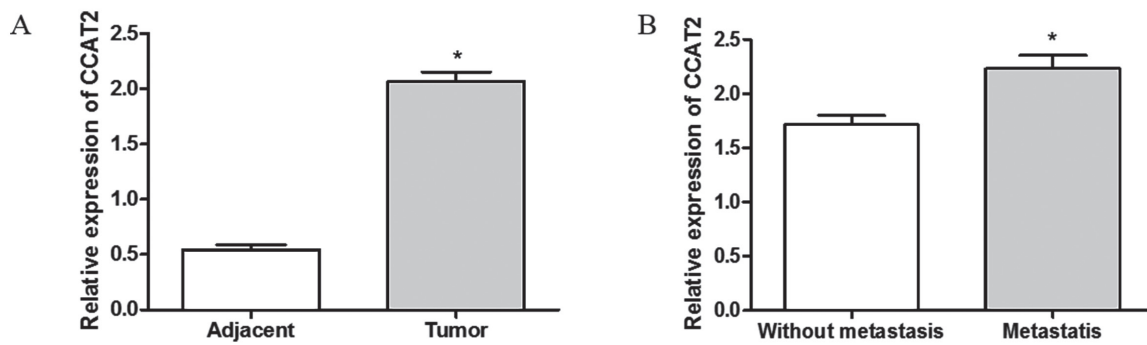


Figure 1. The relative expression level of CCAT2 in adjacent non-cancerous and breast cancer tissues without or with metastasis. (A) The relative mRNA expression level of CCAT2 in adjacent non-cancerous and breast cancer tissues was analyzed by qRT-PCR. (B) The relative mRNA expression level of CCAT2 in breast cancer without or with metastasis was analyzed by qRT-PCR. Data were Mean \pm SD for three independent experiments (* p < 0.05 compared with adjacent non-cancerous group or breast cancer without metastasis group).

The Relative Expression Level of CCAT2 in Breast Cancer Cell Lines

HCC1937 cells are derived from healthy breast tissue and included here as a control. The expression level of CCAT2 in all of the three breast cancer cell lines, including LCC9, MDA-MB-231 and MCF-7 were detected by qRT-PCR and Western blot. We found that the relative mRNA expression levels of CCAT2 were increased in breast cancer cell lines, compared with HCC1937 cell line (Figure 2A). Meanwhile, all cell lines showed higher CCAT2 protein expression levels in breast cancer cell lines as compared with HCC1937 cell line (Figure 2B-C).

The Relative Expression Level of CCAT2 in Breast Cancer Cell Lines After Infected with si-NC or si-CCAT2

To investigate the role of CCAT2 in breast cancer carcinogenesis, a si-CCAT2 was intro-

duced into breast cancer cells. The relative mRNA expression level of CCAT2 in breast cancer cell lines after infected with si-NC or si-CCAT2 was analyzed. The qRT-PCR analysis showed that the relative mRNA expression level of CCAT2 was significantly decreased in breast cancer cells infected with si-CCAT2 compared with HCC1937 cell line (Figure 3A). Western blot analysis showed that si-CCAT2 also down-regulated the protein expression of CCAT2 in breast cancer cells compared with HCC1937 cell line (Figure 3B-C).

Down-regulation of CCAT2 Inhibited the Proliferation in Breast Cancer Cell Lines

The effects of CCAT2 in breast cancer cell proliferation were examined using the MTT assay to identify CCAT2 as a promotor in breast cancer. LCC9 and MCF-7 cells were infected with si-NC or si-CCAT2. Down-regulation of CCAT2 sig-

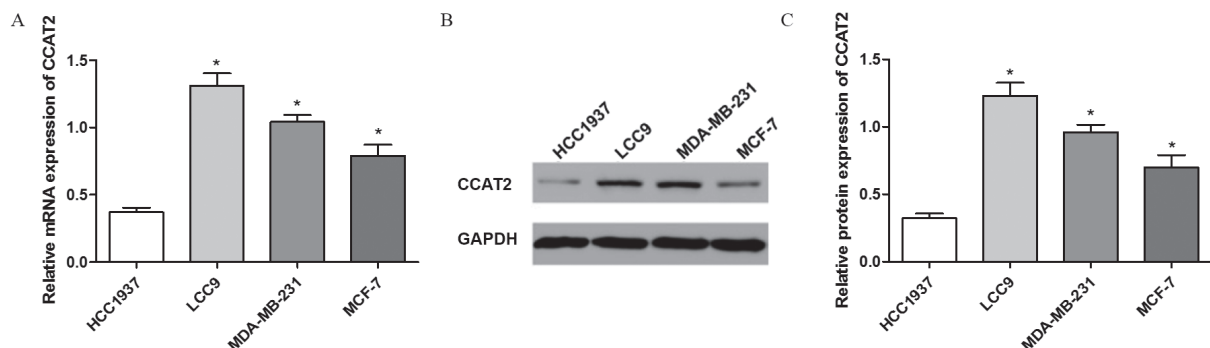


Figure 2. The relative expression level of CCAT2 in breast cancer cell lines. (A) The relative mRNA expression level of CCAT2 in breast cancer cell lines was analyzed by qRT-PCR. (C and D) The relative protein expression level of CCAT2 in breast cancer cell lines were analyzed by Western blot. Data were Mean \pm SD for three independent experiments (* p < 0.05 compared with HCC1937 group).

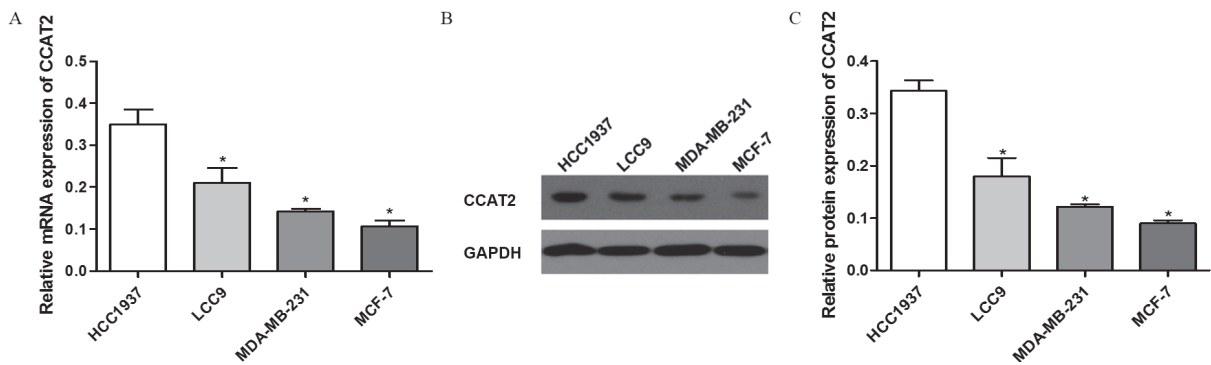


Figure 3. The relative expression level of CCAT2 in breast cancer cell lines after infected with si-NC or si-CCAT2. **A,** The relative mRNA expression level of CCAT2 in breast cancer cell lines after infected with si-NC or si-CCAT2 was analyzed by qRT-PCR. **(C and D)** The relative protein expression level of CCAT2 in breast cancer cell lines after infected with si-NC or si-CCAT2 were analyzed by Western blot. Data were Mean \pm SD for three independent experiments (* p < 0.05 compared with HCC1937 group).

nificantly suppressed the proliferation in breast cancer cells at 72 h and 96 h (Figure 4A-B). These data suggested that down-regulation of CCAT2 inhibited the proliferation of breast cancer cells.

Down-regulation of CCAT2 Inhibited the Invasion and Migration in Breast Cancer Cell Lines

The further experiments were used to confirm the promoter function of CCAT2 in breast cancer. Invasion and migration activities of LCC9 and MCF-7 cells after infected with si-NC or si-CCAT2 were measured with Transwell. The results demonstrated that down-regulation of CCAT2 inhibited the invasion (Figure 5A) and migration (Figure 5B) in breast cancer cell lines compared with si-NC group.

The Effect of Down-regulation CCAT2 on Cell Cycle and Apoptosis in Breast Cancer Cell Lines

The cell cycle distribution and apoptosis rate in LCC9 and MCF-7 cells after infected with si-NC or si-CCAT2 were measured by flow cytometry to study the functional mechanism of CCAT2 in breast cancer. The results demonstrated that cell cycle was prominently shifted from S phase and G2/M phase to G0/G1 phase, cell percentage in G0/G1 phase was significantly increased while cell percentage in S phase was significantly decreased. Cell apoptosis was promoted after infected with si-CCAT2 (Figure 6A-B). These data showed that down-regulation CCAT2 arrested cell cycle in G0/G1 phase to induce cell apoptosis.

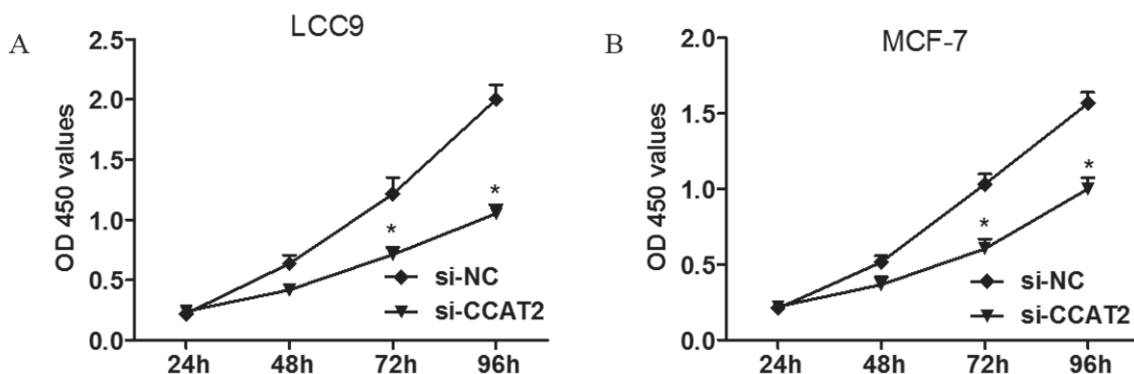


Figure 4. Down-regulation of CCAT2 inhibited the proliferation in LCC9 and MCF-7 cell lines. **A,** LCC9 cells proliferation rate were detected using the CCK8 assay after infected with si-NC or si-CCAT2. **B,** MCF-7 cells proliferation rate were detected using the CCK8 assay after infected with si-NC or si-CCAT2. Data were Mean \pm SD for three independent experiments (* p < 0.05 compared with si-NC group).

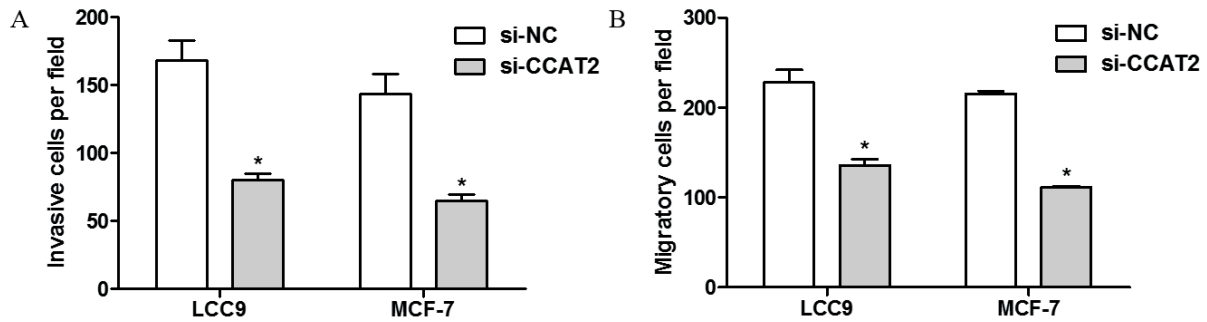


Figure 5. Down-regulation of CCAT2 inhibited the invasion and migration in LCC9 and MCF-7 cell lines. **A**, LCC9 and MCF-7 cells were infected with si-NC or si-CCAT2. Invasion activities of LCC9 and MCF-7 cells were measured with the Transwell. **B**, LCC9 and MCF-7 cells were infected with si-NC or si-CCAT2. Migration activities of LCC9 and MCF-7 cells were measured with the Transwell. Data were Mean \pm SD for three independent experiments (* p < 0.05 compared with si-NC group).

Down-regulation of CCAT2 Inhibited the Activation of TGF- β Signaling Pathway in LCC9 and MCF-7 Cell Lines

To study the mechanism, the relative protein expression level of TGF- β , Smad2 and α -SMA in LCC9 cells after infected with si-NC or si-CCAT2 were analyzed. As it was shown, down-regulation of CCAT2 significantly down-regulated the protein expression levels of TGF- β , Smad2 and α -SMA in breast cancer cells compared with si-NC group (Figure 7A-B). These data showed that down-regulation of CCAT2 inhibited the proliferation, invasion and migration and promoted cell apoptosis in breast cancer cells by regulating TGF- β signaling pathway.

Discussion

In our study, the expression of CCAT2 was upregulated in LCC9, MDA-MB-231, and MCF-7 cells compared with normal human breast cell line HCC1937. Meanwhile, the relative mRNA expression level of CCAT2 in breast cancer tissues was significantly increased compared with adjacent non-cancerous tissues and the expression level of CCAT2 in breast cancer without metastasis was decreased compared with breast cancer metastasis, which showed CCAT2 could promote the development and metastasis in breast cancer patients. Moreover, down-regulation of CCAT2 inhibited the proliferation, invasion and migration in breast

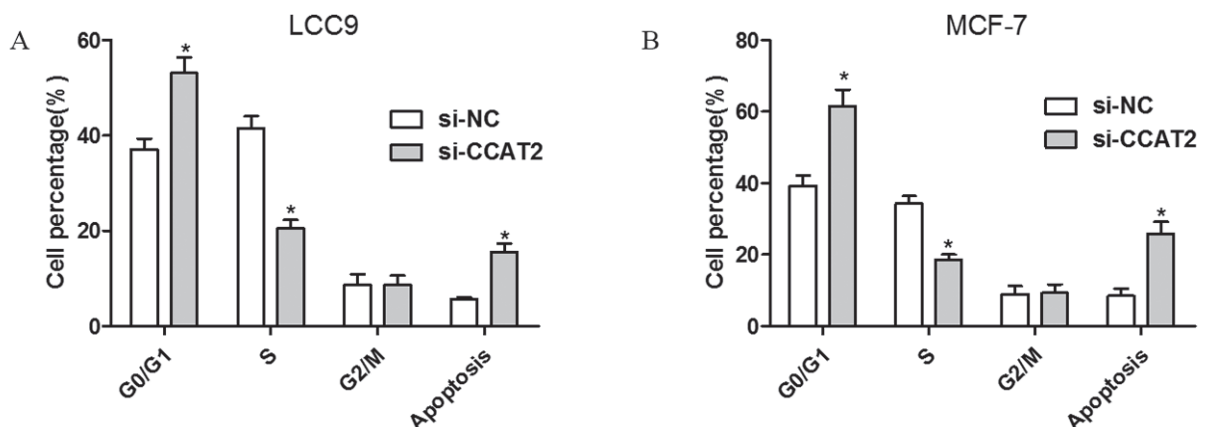


Figure 6. The effect of down-regulation CCAT2 on cell cycle and apoptosis in LCC9 and MCF-7 cell lines. **A**, Flow cytometry analysis was used to detect the cell cycle distribution and apoptosis rate in LCC9 cells after infected with si-NC or si-CCAT2. **B**, Flow cytometry analysis was used to detect the cell cycle distribution and apoptosis rate in MCF-7 cells after infected with si-NC or si-CCAT2. Data were Mean \pm SD for three independent experiments (* p < 0.05 compared with si-NC group).

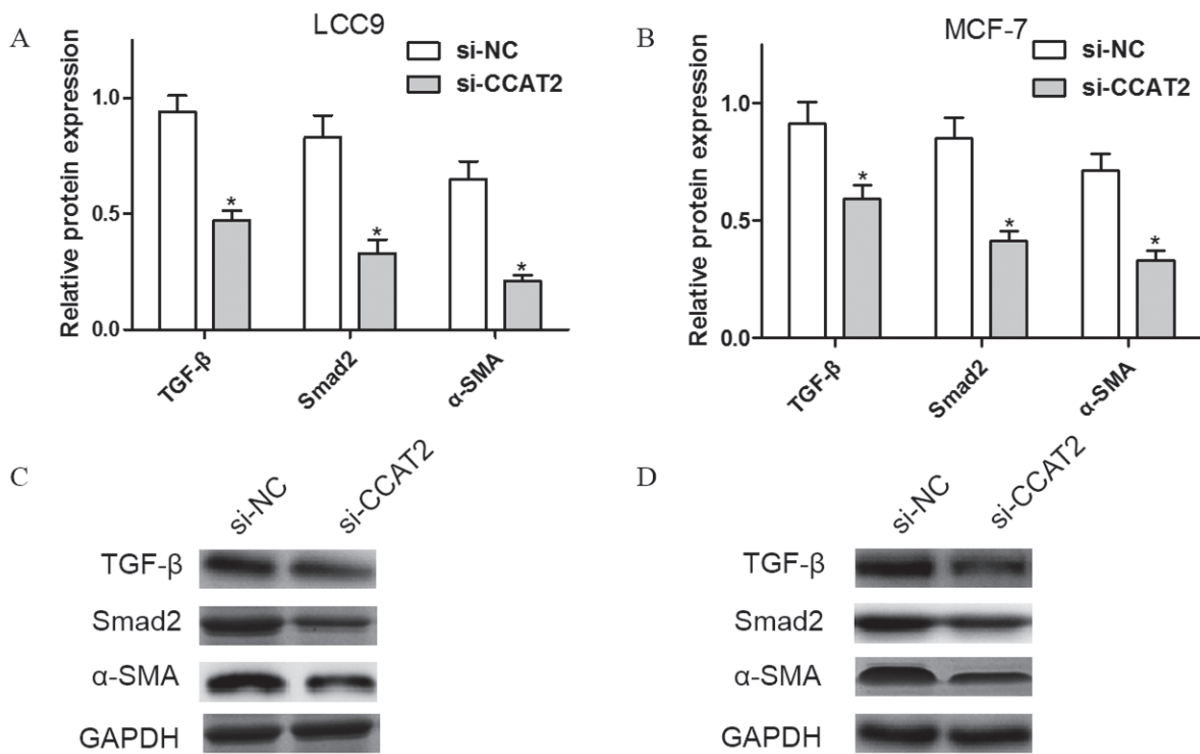


Figure 7. Down-regulation of CCAT2 inhibited the activation of TGF- β signaling pathway in LCC9 and MCF-7 cell lines. **(A and C)** The relative protein expression level of TGF- β , Smad2 and α -SMA in LCC9 cells after infected with si-NC or si-CCAT2 were analyzed by Western blot. **(B and D)** The relative protein expression level of TGF- β , Smad2 and α -SMA in MCF-7 cells after infected with si-NC or si-CCAT2 were analyzed by Western blot. Data were Mean \pm SD for three independent experiments (* $p < 0.05$ compared with si-NC group).

cancer cells. Furthermore, down-regulation of CCAT2 caused breast cancer cell cycle prominently shifted from S phase and G2/M phase to G0/G1 phase, cell percentage in G0/G1 phase was significantly increased while cell percentage in S phase was significantly decreased and cell apoptosis was promoted. In conclusion, down-regulation of CCAT2 significantly down-regulated the protein expression levels of TGF- β , Smad2 and α -SMA in breast cancer cells. Our observations suggest that CCAT2 might serve as a tumor promotor in breast cancer and provide novel target for the treatment. Accumulating evidence¹⁴⁻¹⁶ has shown specific binding and significant secondary structures were involved in multiple gene regulatory networks. Several researches^{17,18} have reported lncRNAs with biological functions associated with the proliferation, metastasis, invasion, migration and apoptosis in human cancer cells. Up to now, well-known discovered ncRNAs are rRNAs, tRNAs, piRNAs, miRNAs and lncRNAs. It makes us wonder if the lncRNA

also correlate with the cancer diagnosis and prognosis. Studies showed CCAT2 was up-regulated in human gastric cancer and colorectal cancer. Up-regulated CCAT2 promoted tumor growth, metastasis and could work as a tumor promotor or impose long-range modulation on the expression of cell cycle-related genes^{26,27}. Lan et al²⁸ reported overexpression of CCAT2 promoted gastric cancer cell migration and invasion by regulating the MMPs and key EMT markers. However, the predictive application of CCAT2 in breast cancer metastasis and prognosis needs further research. In this study, the expression of CCAT2 was confirmed to be higher in tumor tissues. Down-regulation of CCAT2 inhibited cell proliferation, invasion and migration. The results indicated that CCAT2 could affect the growth and mobility of breast cancer. TGF- β signaling pathway plays an important role in promoting EMT, thereby contributing to increased tumor invasion²⁹. The TGF- β -SMAD pathway enhances melanoma progression by controlling different stages in

the process of cancer cell metastasis, including epithelial-to-mesenchymal transition (EMT)³⁰. Abnormal EMT transformation enhances tumor cells migration from the primary site into circulation³¹. Several key regulators are implicated in the process of EMT, including Smad2, Snail, Slug. Enhanced TGF- β expression has been reported to induce vessel invasion, metastasis, advanced tumor stages and shorter survival times in-patient with melanoma^{32,33}. In contrast, the suppression of TGF- β signaling is effective for the suppression of cell migration in the mouse model^{34,35}. In this paper, down-regulation of CCAT2 inhibited the proliferation, invasion and migration in breast cancer cells and down-regulation of CCAT2 caused breast cancer cell cycle prominently shifted from S phase and G2/M phase to G0/G1 phase. Moreover, cell percentage in G0/G1 phase was significantly increased while cell percentage in S phase was significantly decreased. Also, down-regulation of CCAT2 promoted cell apoptosis. To characterize the mechanism of CCAT2 in breast cancer, the relative protein expression level of TGF- β , Smad2 and α -SMA in LCC9 cells after infected with si-NC or si-CCAT2 were analyzed. Mechanistically, down-regulation of CCAT2 significantly down-regulated the protein expression levels of TGF- β , Smad2 and α -SMA in breast cancer cells compared with si-NC group. These data showed that down-regulation of CCAT2 inhibited the proliferation, invasion and migration and promoted cell apoptosis in breast cancer cells by regulating TGF- β signaling pathway.

Conclusions

CCAT2 was highly expressed in breast cancer. Down-regulation of CCAT2 inhibited the proliferation, invasion and migration and promoted cell apoptosis in breast cancer cells by regulating TGF β signaling pathway.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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