

Long non-coding RNA CCAT1 enhances human non-small cell lung cancer growth through downregulation of microRNA-218

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Abstract. Long non-coding RNAs (lncRNAs) have critical functions in non-small cell lung cancer (NSCLC) growth. In the present study, we showed that lncRNA-CCAT1 was upregulated in NSCLC tissues. High expression of lncRNA-CCAT1 was related to tumor growth and reduced survival rate. We used short hairpin RNAs (shRNAs) to inhibit the expression of lncRNA-CCAT1 in NSCLC cells. *In vitro* and *in vivo* results demonstrated that lncRNA-CCAT1 knockdown suppressed tumor proliferation and induced apoptosis. Furthermore, microRNA-218 (miR-218) was confirmed as an effective target of lncRNA-CCAT1 in NSCLC. B lymphoma Mo-MLV insertion region 1 homolog (BMI-1), which served as a downstream target of miR-218, was also inhibited by lncRNA-CCAT1 knockdown. In conclusion, the present study indicated that upregulation of lncRNA-CCAT1 in NSCLC is associated with tumor malignant potential. lncRNA-CCAT1 enhances tumor growth in NSCLC by directly inhibiting miR-218 and indirectly increasing BMI-1 expression.

Introduction

Rapid growth is one of the main characteristics of non-small cell lung cancer (NSCLC) (1). Patients burdened with large size tumors usually have poor clinical prognosis. Even though new drugs targeting various critical oncogenes significantly reduce tumor size before surgery, side-effects seriously restrict the effective clinical application of these drugs (2). Thus, the identification of new therapeutic targets remains vital for effective NSCLC treatment.

Long non-coding RNAs (lncRNAs) are a group of evolutionarily conserved RNAs that are >200 nucleotides in length

with no protein-coding capacity (3). Recent studies have shown that lncRNAs play critical roles in various biological processes including proliferation, invasion and angiogenesis. lncRNA-XIST was found to be upregulated in lung cancer cells and accelerated lung cancer cell growth through targeting miR-140 (4). lncRNA-PVT1 directly interacted with miR-200a and miR-200b to facilitate NSCLC cell invasion by upregulating MMP9 expression (5). Inhibition of lncRNA-p21 was found to induce global downregulation of the expression of angiogenesis-related genes including VEGFA in human NSCLC (6).

Recently, lncRNA-CCAT1 (simply CCAT1) was demonstrated to promote tumor growth and metastasis in human cancers (7). In osteosarcoma, CCAT1 was found to be upregulated in osteosarcoma tissues and cell lines (8). High levels of CCAT1 promoted cell proliferation and migration by inhibiting miR-148. CCAT1 was also found to be upregulated in renal cell carcinoma (RCC) (9). CCAT1 knockdown inhibited cell viability and increased apoptosis of RCC cells *in vitro*. Overexpression of CCAT1 could bind miR155-5p and let7b-5p to account for the poor prognosis of human oral squamous cell carcinoma (10). However, the expression and biological functions of CCAT1 in NSCLC are still unclear.

In the present study, we determined the expression of CAAT1 in NSCLC tissues and cell lines. Upregulation of CAAT1 was associated with large tumor size and poor prognosis in NSCLC patients. CCAT1 knockdown inhibited NSCLC tumor growth *in vitro* and *in vivo* by rescuing the expression of miR-218 and simultaneously inhibiting the expression of BMI-1, which is a downstream target of miR-218.

Materials and methods

Clinical samples and cell lines. Fifty paired NSCLC and matched tumor-adjacent tissues were collected from Linyi Central Hospital (Linyi, China) and North China University of Science and Technology Affiliated Hospital (North China University of Science and Technology, China). These patients included 31 males and 19 females who received surgical resection between March 2011 and January 2013. The age range was between 41 and 73 years. The use of clinical data was approved by the Biomedical Ethics Committee of Linyi Central Hospital. All patients enrolled in this study signed

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an informed consent. Normal lung epithelial BEAS-2B cells and 4 NSCLC cell lines (A549, H1299, H1975 and HCC827) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 1% penicillin and 1% streptomycin.

Real-time quantitative reverse transcription-PCR (qRT-PCR). Total RNA from tissues and cells was isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). One-Step Perfect Real-Time RT-qPCR (SYBR-Green I) kit (Takara Biotechnology Co., Ltd., Dalian, China) was used to detected the expression levels of lncRNA-CCAT1, miR-218 and BMI-1 mRNA. miR-218 and RNU6 Bulge-Loop™ primers were purchased from RiboBio Co., Ltd. (Guangzhou, China). lncRNA-CCAT1, BMI-1 and GAPDH primers were synthesized by Sangon Co., Ltd. (Shanghai, China). Data were analyzed using the $2^{-\Delta\Delta C_q}$ method (11). The sequences of primers used are shown as follows: lncRNA-CCAT1 (sense, 5'-AGA AACACTATCACCTACGC-3' and antisense, 5'-CTTAAC AGGGCATTGCTAACATCT-3'); BMI-1 (sense, 5'-GTGCTTTGT GGAGGGTACTTCAT-3' and antisense, 5'-TTGGACATCACA AATAGGACAATACTT-3'); GAPDH (sense, 5'-CCAGGG CTGCTTTAACTCT-3' and antisense, 5'-GGACTCCAC GACGTACTCA-3'). The thermocycling conditions were: holding stage, 50°C for 2 min, 95°C for 10 min; PCR stage (40 times), 95°C for 15 sec, 60°C for 1 min.

Cell infection and transfection. The control short hairpin RNA (shRNA) (sh-ctrl) plasmids and CCAT1-specific shRNA (sh-CCAT1) plasmids were purchased from GeneCopoeia (Guangzhou, China) and cloned into lentivirus vectors. Lentiviruses were packaged and amplified in 293T cells (ATCC) according to the manufacturer's instructions. Lentivirus supernatant was used to infect the tumor cells. The effect of infection was determined by qRT-PCR. The negative control inhibitor (anti-miR-ctrl) and miR-218 inhibitor (anti-miR-218) were purchased from Riobio Co., Ltd. (Guangzhou, China). These inhibitors were transfected into tumor cells using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) reagent. Cells were used for further experiments after 24-h transfection.

Cell Counting Kit-8 (CCK-8) cell proliferation assay. For cell proliferation detection, we used CCK-8 assay according to the manufacturer's recommendations (Sangon Biotech Co., Ltd., Shanghai, China). Infected or transfected cells were cultured in a 96-well plate at a density of 2,000 cells/well in quintuplicate. CCK-8 solution (10 µl) was added to each well plate and was incubated for 4 h in an incubator. Cells were incubated at 37°C for 4 h. The absorbance at 450 nm was measured by a microplate reader.

Cell apoptosis assay. To evaluate the apoptosis of NSCLC cells, flow cytometric assay was performed to evaluate the percentage of apoptotic cells. In brief, cells were harvested and re-suspended in phosphate-buffered saline (PBS) solution, and then stained with Annexin V-FITC/PI detection kit

(BD Biosciences, San Jose, CA, USA) and subjected to FACS analysis (FlowJo, v7.60; FlowJo LLC, Ashland, OR, USA).

In vivo experiments. Ten BALB/cA, 4-week-old, 16-18 g, female nude mice were obtained from the Animal Center of Jining Medical College. The mice were housed in sterilized cages under appropriate environmental conditions (25°C, 45% humidity), were fed a regular chow diet with water *ad libitum*, and were handled according to the requirements of the National Institutes of Health guidelines for care and use of laboratory animals. All animal protocols were approved by the Biomedical Ethics Committee of Linyi Central Hospital. Mice were randomly divided into 2 groups. Recombinant A549 and H1975 cells were subcutaneously injected into the nude mice to investigate the functions of CCAT1 for tumor growth. The mice were sacrificed after 4 weeks by CO₂ euthanasia (the flow rate of CO₂ was 20% displacement/min). Final tumor volumes were calculated based on the following formula: (length x width²)/2.

Ki-67 immunohistochemical staining. Subcutaneous tumor tissues were made into paraformaldehyde-fixed paraffin sections. Sections were processed with xylene, alcohol and PBS, sequentially. Sections were incubated with Ki-67 primary antibodies [cat. no. 9027; Cell Signaling Technology (CST), Inc., Danvers, MA, USA] (1:100) at 4°C overnight. Biotinylated secondary antibodies were used to combine Ki-67 antibodies, detected by HRP-streptavidin conjugates and visualized by DAB. The Ki-67 index was calculated according to a previous study (12).

Western blotting. Total cell proteins were extracted with RIPA buffer, separated on 10% SDS-PAGE gel and transferred onto a nitrocellulose membrane (Invitrogen; Thermo Fisher Scientific, Inc.). The membranes were incubated with the following primary rabbit anti-human antibodies purchased from (CST) at 4°C overnight: BMI-1 (cat. no 6964; dilution at 1:1,000) and GAPDH (cat. no 5174; dilution at 1:1,000). Then, the membranes were incubated with HRP-linked goat anti-rabbit antibodies (cat. no 7074; dilution at 1:5,000; CST), and the signals were detected using the Bio-Rad Gel imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. All quantitative data are expressed as mean ± SD. The Statistical Product and Service Solutions v21.0 software (SPSS 21.0; IBM Corp., Armonk, NY, USA) was used to perform statistical analysis. The Pearson's Chi-square test was used to analyze the relationship between CCAT1 expression and clinical features. Student's t-test was performed for data in 2 groups. ANOVA was used to analyze the data in 3 or more groups and Bonferroni's method was used as a post hoc test. Overall survival and progression-free survival analysis were performed using the Kaplan-Meier method for plotting and the log-rank test for comparison. P<0.05 indicated a statistically significant difference.

Results

CCAT1 is upregulated in NSCLC tissues and is associated with poor patient prognosis. As shown in Fig. 1A, the expression of CCAT1 was elevated in NSCLC tissues (P<0.001).

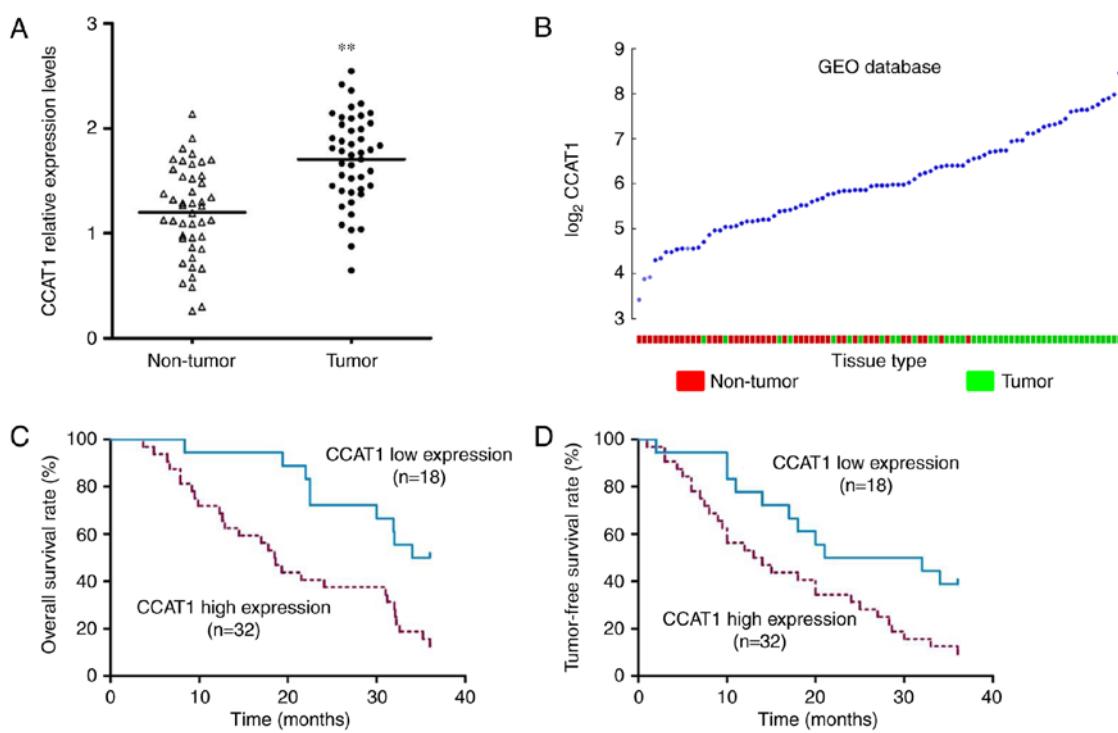


Figure 1. Expression and prognostic significance of CCAT1 in NSCLC. (A) CCAT1 is upregulated in NSCLC tissues. **P<0.01, compared with non-tumor tissues. (B) NSCLC tissues from GEO database showed high expression of CCAT1. Patients with high expression levels of CCAT1 had a low 3-year overall survival rate (C) and tumor-free survival rate (D) after surgery. NSCLC, non-small cell lung cancer; GEO, Gene Expression Omnibus.

Table I. Correlation between CCAT1 and clinical features in NSCLC (n=50).

Clinical characteristics	Total	CCAT1 expression		χ^2	P-value
		High n=32	Low n=18		
Sex					
Male	31	22	9	1.719	0.190
Female	19	10	9		
Age (years)					
<50	21	11	10	2.122	0.145
≥50	29	21	8		
Smoking status					
Yes	26	19	7	1.937	0.164
No	24	13	11		
Tumor size (cm)					
>3	25	20	5	5.556	0.018 ^a
≤3	25	12	13		
Lymphatic metastasis					
Present	30	22	8	2.836	0.092
Absent	20	10	10		
TNM stage					
I+II	26	14	12	2.424	0.119
III+IV	24	18	6		

NSCLC, non-small cell lung cancer; TNM, tumor-node-metastasis.

^aP<0.05.

Furthermore, consistent with our results, the expression of CCAT1 was also increased in 91 NSCLC tissues available in Gene Expression Omnibus (GEO) database (GSE18842) (13) (Fig. 1B). To further investigate the clinical significance of CCAT1, we used the mean expression value of CCAT1 (=1.804) to divide these 50 patients into 2 subgroups: CCAT1 high expression group (n=32) and CCAT1 low expression group (n=18). As shown in Table I, high expression of CCAT1 was associated with large tumor size (P=0.018). Moreover, patients in the high CCAT1 expression group had poor 3-year overall survival rate (Fig. 1C, P=0.003, HR=2.736) and tumor-free survival rate (Fig. 1D, P=0.014, HR=2.224).

CCAT1 knockdown inhibits proliferation and induces apoptosis in NSCLC cells. Next, compared with BEAS-2B cells, we also found that the expression levels of CCAT1 were upregulated in all NSCLC cell lines (Fig. 2A, P<0.05, respectively). We used shRNA to knock down CCAT1 expression in A549 and H1975 cells, which were two cell lines that expressed the highest CCAT1 levels (Fig. 2B, P<0.01, respectively). CCK-8 assays showed that CCAT1 knockdown significantly decreased cell viability in the two cell lines (Fig. 2C, P<0.05, respectively). Furthermore, CCAT1 knockdown also decreased colony formation number of A549 and H1975 cells (Fig. 2D, P<0.05, respectively). On the contrary, flow cytometry (FCW) assays demonstrated that CCAT1 knockdown increased the percentage of apoptotic NSCLC cells (Fig. 2E, P<0.05, respectively).

CCAT1 knockdown inhibits tumor growth in vivo. When we demonstrated that CCAT1 knockdown inhibited NSCLC cell proliferation *in vitro*, we also utilized a subcutaneous xenograft growth model to investigate the growth promoting functions of

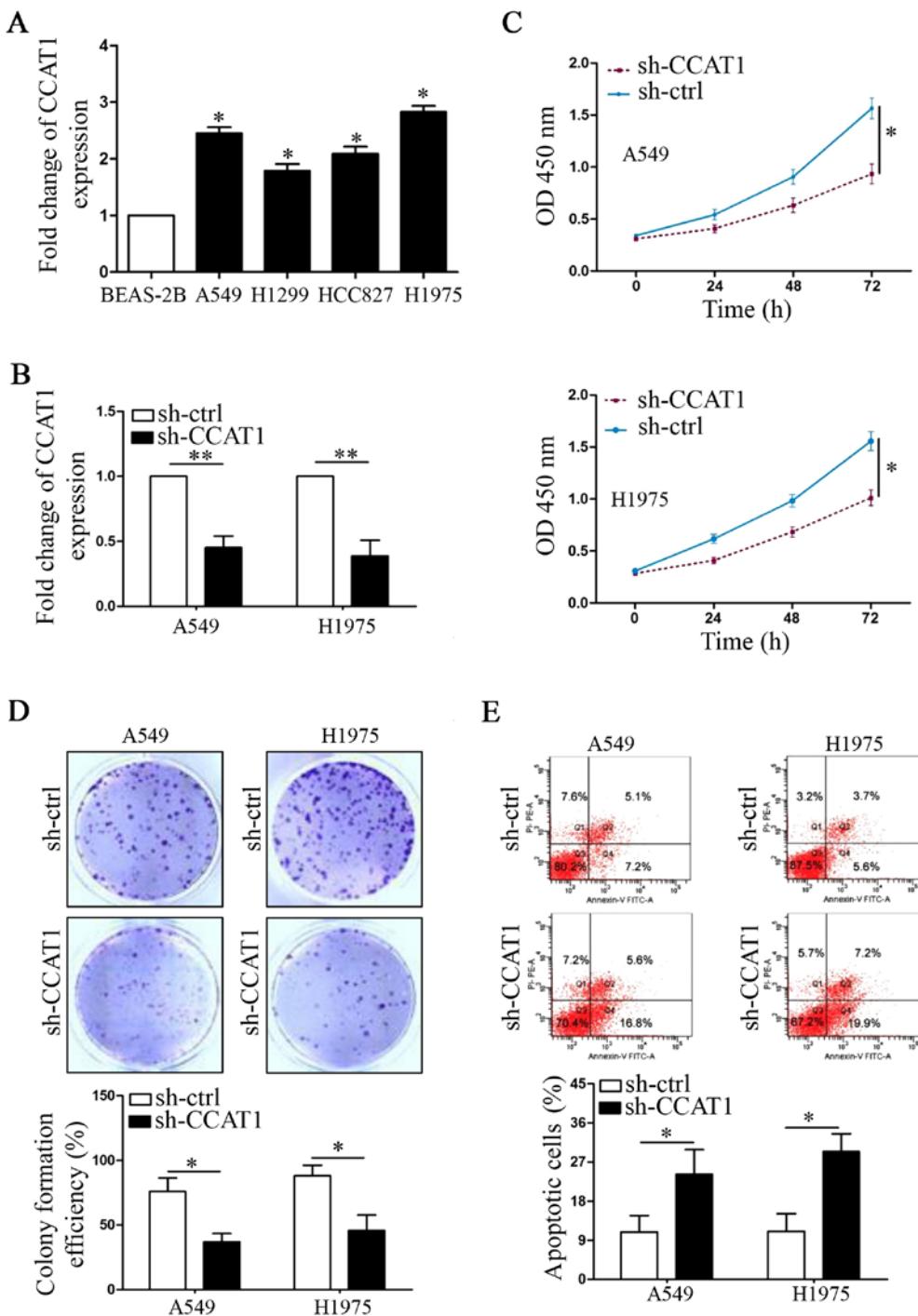


Figure 2. CCAT1 knockdown inhibits the proliferation and induces the apoptosis of NSCLC cells *in vitro*. (A) CCAT1 expression levels were elevated in NSCLC cell lines. *P<0.05, compared with BEAS-2B cells. (B) The expression levels of CCAT1 were downregulated by shRNA in A549 and H1975 cells. **P<0.01, compared with the sh-ctrl group. (C) The viability of A549 and H1975 cells were inhibited by CCAT1 knockdown. *P<0.05, compared with the sh-ctrl group. (D) CCAT1 knockdown decreased the colony formation number of the A549 and H1975 cells. *P<0.05, compared with the sh-ctrl group. (E) CCAT1 knockdown induced the apoptosis of A549 and H1975 cells. *P<0.05, compared with the sh-ctrl group. NSCLC, non-small cell lung cancer.

CCAT1 *in vivo*. As shown in Fig. 3A, downregulation of CCAT1 inhibited the tumor growth in nude mice (P<0.05, respectively). This effect was further confirmed by Ki-67 immunohistochemical staining in mouse tumor tissue sections (Fig. 3B, P<0.05, respectively). These data suggest that lncRNA-CCAT1 promotes tumor growth of NSCLC *in vitro* and *in vivo*.

CCAT1 exerts its function by targeting the miR-218/BMI-1 axis in NSCLC cells. miR-218 was predicted as a potential

interactive target of CCAT1 by informatic analysis (MiRanda: <http://www.microrna.org/>). The following evidence was used to demonstrate that CCAT1 exerted its function by downregulating miR-218 in NSCLC cells. First, qRT-PCR results showed that the expression of miR-218 was upregulated in CCAT1-knockdown A549 and H1975 cells (Fig. 4A, P<0.01 and P<0.001, respectively). Second, miR-218 inhibitors were used to repress its expression in CCAT1-knockdown cells (Fig. 4B, P<0.05, respectively). CCK-8 and FCW assays

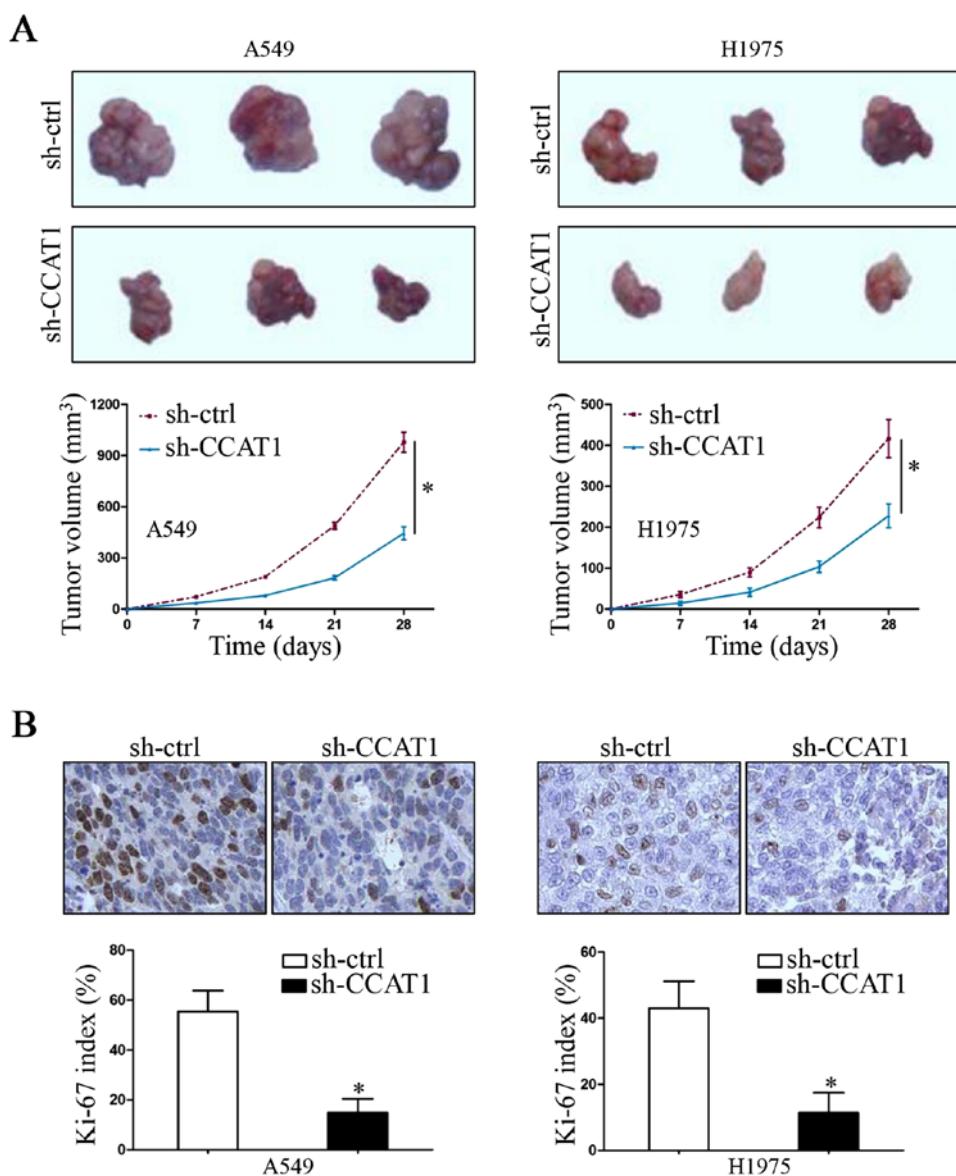


Figure 3. CCAT1 knockdown inhibits NSCLC cell growth *in vivo*. (A) Downregulation of CCAT1 decreased the volume of tumors formed by A549 and H1975 cells subcutaneously injected in nude mice. *P<0.05, compared with the sh-ctrl group. (B) Downregulation of CCAT1 decreased the percentage of Ki-67-positive cells in mouse tumor sections. *P<0.05, compared with the sh-ctrl group. NSCLC, non-small cell lung cancer.

were performed to show that the CCAT1 knockdown-induced anti-growth effect was abrogated by downregulation of miR-218 (Fig. 4C and D, P<0.05, respectively). BMI-1 was reported as a downstream target of miR-218 in NSCLC. Finally, we detected the mRNA and protein levels of BMI-1 in NSCLC cells. As shown in Fig. 5A, both mRNA and protein levels of BMI-1 were decreased by CCAT1 knockdown (P<0.05, respectively). Moreover, the expression of BMI-1 was rescued in CCAT1-knockdown cells by inhibition of miR-218 (Fig. 5B, P<0.05, respectively). Thus, these results may partly explain the anti-growth mechanisms of lncRNA-CCAT1 knockdown in NSCLC.

Discussion

Tumor growth is an important risk factor for the poor prognosis of NSCLC patients. Numerous studies have demonstrated that long non-coding RNAs (lncRNAs) function as tumor growth

promotors or inhibitors in NSCLC (14,15). Long non-coding RNA MEG3 was found to inhibit NSCLC cell proliferation, arrest the cell cycle and promote apoptosis by inducing p53 expression (16). Moreover, decreased expression of MEG3 was also associated with advanced pathologic stage and tumor size in NSCLC patients (17). However, long non-coding RNA ANRIL was found to be upregulated in NSCLC tissues and cell lines (18). ANRIL promoted cell proliferation and suppressed apoptosis by silencing KLF2 and P21 expression. In the present study, our results showed that lncRNA-CCAT1 is overexpressed in NSCLC tissues and cell lines. Importantly, elevated expression of CCAT1 was also confirmed in a GEO database including 91 NSCLC patients. Moreover, high expression of CCAT1 was related to large tumor size and short survival time after surgery. This prognostic prediction value also exists in breast cancer (19) and gastric carcinoma (20). Taken together, these data indicated that lncRNA-CCAT1 may play a critical role in the progression of NSCLC.

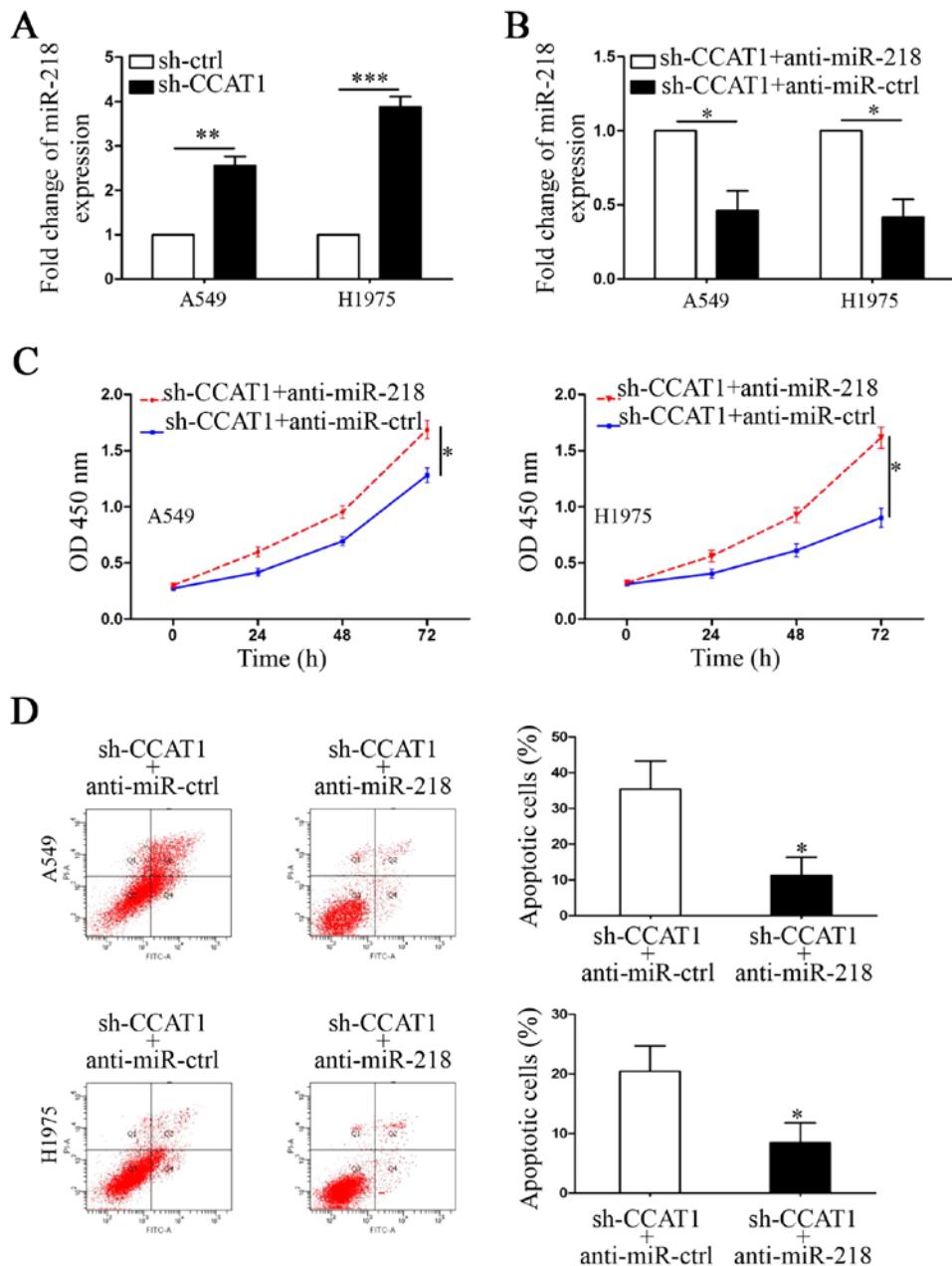


Figure 4. Downregulation of miR-218 reverses the suppression of cell growth in CCAT1-knockdown cells. (A) The expression of miR-218 was elevated in CCAT1-knockdown cells. **P<0.01 and ***P<0.001, compared with the sh-ctrl group. (B) miR-218 inhibitor downregulated miR-218 expression levels. *P<0.05, compared with anti-miR-ctrl group. (C) Downregulation of miR-218 increased the cell viability of the CCAT1-knockdown cells. *P<0.05, compared with the anti-miR-ctrl group. (D) Downregulation of miR-218 inhibited the increase in cell apoptosis induced by CCAT1 knockdown in NSCLC cells. *P<0.05, compared with the anti-miR-ctrl group. miR-218, microRNA-218; NSCLC, non-small cell lung cancer.

Most studies have shown that CCAT1 promotes cell migration and invasion in human cancers, but few studies have investigated its function in tumor growth (21). In the present study, we silenced CCAT1 expression in NSCLC cells. *In vitro* analysis confirmed that CCAT1 knockdown inhibited cell viability and proliferation, and induced apoptosis in NSCLC cells. This tumor growth promotion effect of CCAT1 was also blocked by shRNAs in a nude mouse subcutaneous xenograft model. These results may partly explain why high expression of CCAT1 is associated with large tumor size in NSCLC patients.

lncRNAs usually exert their functions through sponging various microRNAs (22). lncRNA-CCAT1 has been reported to sponge let-7 in hepatocellular carcinoma (23), miR-410

in glioma (24) and miR-181a in nasopharynx cancer (25). In the present study, we used an online database to select a new potential target miRNA of CCAT1 named microRNA-218 (miR-218). Previous research has established the inhibitory role of miR-218 in tumor growth (26). In our CCAT1-knockdown NSCLC cells, upregulation of miR-218 was noted. The cell proliferation was increased whereas apoptosis was inhibited when we used the miR-218 inhibitor to block its expression. That indicates that lncRNA-CCAT1 increases NSCLC tumor growth by sponging miR-218.

B lymphoma Mo-MLV insertion region 1 homolog (BMI-1) is a component of polycomb repressive complex 1 (PRC1) (27). In NSCLC, BMI-1 has been demonstrated as an oncogene due

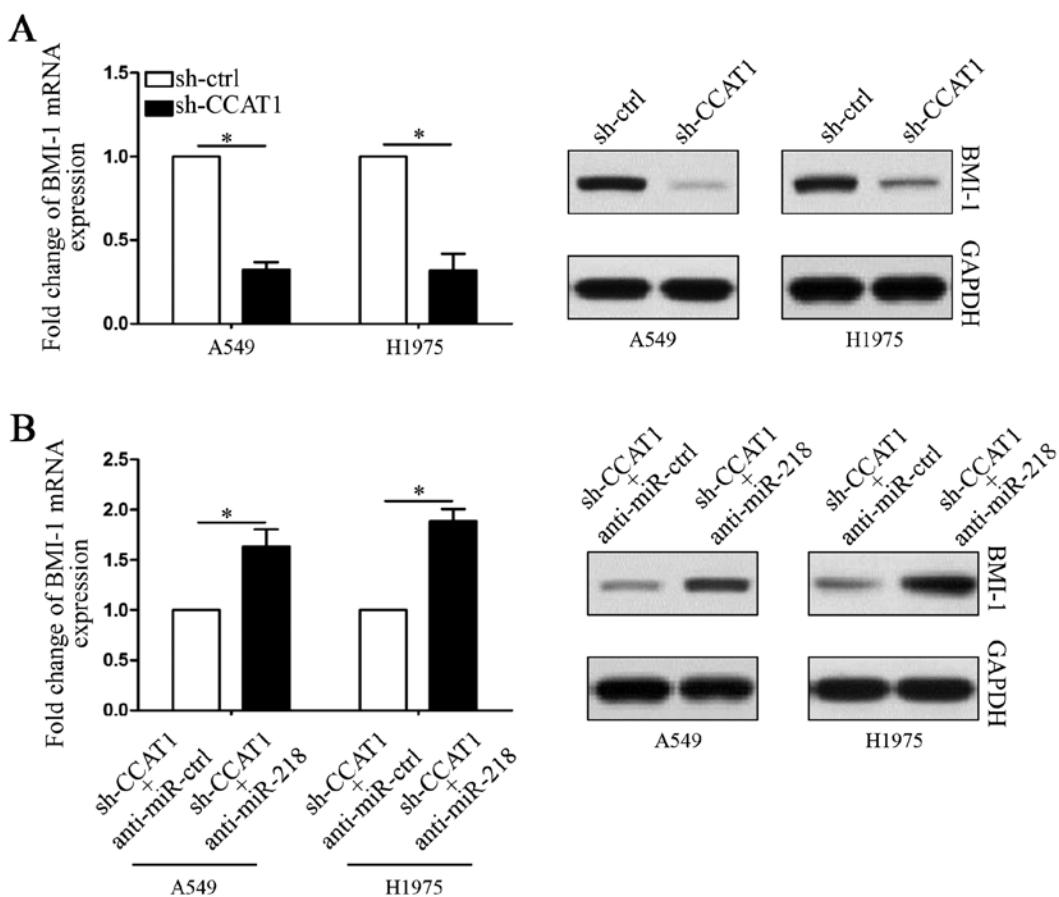


Figure 5. BMI-1 is a downstream target of the CCAT1/miR-218 axis in NSCLC cells. (A) CCAT1 knockdown in A549 and H1975 cells significantly decreased BMI-1 mRNA and protein expression levels. *P<0.05, compared with the sh-ctrl group. (B) Downregulation of miR-218 increased BMI-1 mRNA and protein expression in CCAT1-knockdown cells. *P<0.05, compared with the anti-miR-ctrl group. BMI-1, B lymphoma Mo-MLV insertion region 1 homolog; NSCLC, non-small cell lung cancer.

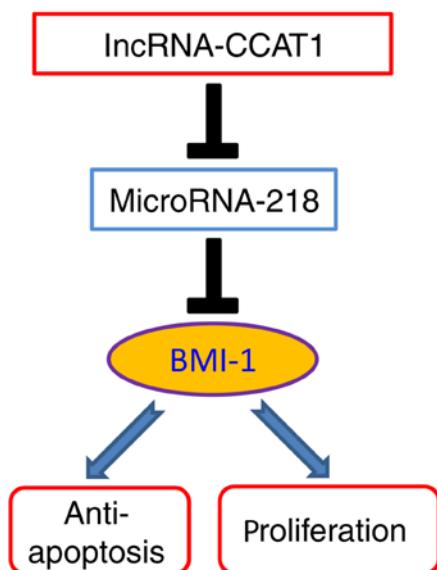


Figure 6. Proposed model for regulation of NSCLC tumor growth through the CCAT1/miR-218/BMI-1 signaling pathway. NSCLC, non-small cell lung cancer; BMI-1, B lymphoma Mo-MLV insertion region 1 homolog.

to its functions in tumor proliferation, invasion and chemo-resistance (28,29). In the present study, it was found that the

expression of BMI-1 was downregulated by CCAT1 silencing in NSCLC cells. On the contrary, the expression levels of BMI-1 were reversed in the CCAT1-knockdown NSCLC cells by inhibiting miR-218 expression. Thus, we conclude that CCAT1 promotes NSCLC growth by increasing BMI-1 expression through decreasing the expression levels of miR-218 (Fig. 6).

In conclusion, we demonstrated that long non-coding RNA CCAT1 is upregulated in NSCLC tissues and cell lines, and its elevated expression is associated with malignant clinical features. IncRNA-CCAT1 promotes NSCLC growth behaviors by targeting the miR-218/BMI-1 axis. These results suggest that IncRNA-CCAT1 is a potential therapeutic target for NSCLC.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

LZ and PM conceived and designed the study. LZ, LW and YW performed the experiments. LZ wrote the paper. LZ, LW, YW and PM reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The use of clinical data was approved by the Biomedical Ethics Committee of Linyi Central Hospital (Linyi, China). Patients enrolled in this study signed an informed consent. All animal protocols were approved by the Biomedical Ethics Committee of Linyi Central Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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