

Overexpression of the long noncoding RNA CCAT1 promotes metastasis via epithelial-to-mesenchymal transition in lung adenocarcinoma

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Abstract. The long noncoding RNA (lncRNA) colon cancer-associated transcript 1 (CCAT1) has been identified as an oncogene in multiple types of human malignancy, and the aberrant expression of CCAT1 has been associated with the tumorigenesis and progression of cancer. However, the underlying mechanism of how CCAT1 affects malignant behaviors in lung adenocarcinoma cells remains unknown. In the current study, the expression of CCAT1 was identified to be increased in lung adenocarcinoma tissues (n=96) by reverse transcription-quantitative polymerase chain reaction (RT-qPCR), and its expression level was associated with epidermal growth factor receptor (EGFR) expression (P=0.011), lymphatic metastasis (P=0.003) and tumor node metastasis (TNM) stage (P=0.003). *In vitro*, by using Transwell assays, the overexpression of CCAT1 was demonstrated to promote the migration and invasion of H358 lung adenocarcinoma cells; while downregulation of CCAT1 expression inhibited H1650 cell migration and invasion. Furthermore, western blot analysis indicated that aberrant CCAT1 expression may induce epithelial-to-mesenchymal transition (EMT) by regulating the expression levels of EMT markers (E-cadherin, N-cadherin and vimentin). In conclusion, these results indicate that CCAT1 is able to promote the metastasis of lung adenocarcinoma cells by inducing EMT.

Introduction

Lung cancer is one of the most common causes of cancer-associated mortality and has amongst the highest morbidity rates of all types of malignancy in China (1). Non-small cell lung cancer (NSCLC) accounts for 80-85% of all lung cancer cases, with lung adenocarcinoma being the most common type (2). Irrespective of the progress made in diagnosis and therapy, the prognosis of patients with lung adenocarcinoma remains poor (2). A primary feature of lung adenocarcinoma is the migration and invasion of neoplasms, which are responsible for the high mortality rate (3). Therefore, understanding the mechanisms underlying metastasis in lung adenocarcinoma may reveal novel therapeutic targets.

Colon cancer-associated transcript 1 (CCAT1), also known as CARLo-5 (onco-lncRNA-40), is a long noncoding RNA (lncRNA) whose gene is located at chromosome 8q24.21, and was originally identified as an oncogene in colon cancer (4). Aberrant expression of CCAT1 in cancer cells significantly affects cell-cycle regulation and tumor development, and CCAT1 expression has been shown to predict the prognosis of patients with various tumor types (5-8). However, little is known about the expression levels of CCAT1 in lung adenocarcinoma or whether this lncRNA is involved in the progression of lung adenocarcinoma.

In the present study, the expression level and the function of CCAT1 in lung adenocarcinoma were investigated. The results demonstrated that CCAT1 was upregulated in lung adenocarcinoma tissues, and promoted the migration and invasion of lung adenocarcinoma cells by inducing epithelial-to-mesenchymal transition (EMT).

Materials and methods

Patients and specimens. Lung adenocarcinoma tissues (n=96, aged 39-80 years; mean, 60.0 years) and pair-matched adjacent normal tissues were obtained from patients diagnosed with lung adenocarcinoma of tumor-node-metastasis (TNM) stage I-III. All patients were diagnosed between August 2010 and August 2011 at The Third Affiliated Hospital of Wenzhou Medical University (Wenzhou, China). The exclusion criteria included previous diagnosis with respiratory system diseases,

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metastasis and patients receiving radiotherapy or chemotherapy prior to their enrollment in the present study. In order to examine tumor cell concentration, frozen sections from all resected fresh tissues underwent histological analyses, and only samples with a tumor cell concentration >60% were included in the present study. Samples were immediately frozen in liquid nitrogen and stored at -80°C until use. Informed consent was obtained from each patient, and the study was approved by the Ethics Committee of The Third Affiliated Hospital of Wenzhou Medical University. Patient data are summarized in Table I.

Cell culture. Human lung cancer cell lines (H358, H23, H1975, H1650, H1299 and A549) and a normal human bronchial epithelial cell line (16HBE) were obtained from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Invitrogen RPMI-1640 medium with 10% fetal bovine serum (FBS) and 100 U/ml penicillin (all from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in humidified air with 5% CO₂.

Reagents and transfection. cDNA of human CCAT1 gene was synthesized by PrimeScript RT Master Mix kit (Takara Bio, Inc., Otsu, Japan). The cDNA encoding CCAT1 was amplified by polymerase chain reaction (PCR) and subcloned into the pcDNA3.1 vector (Invitrogen, Thermo Fisher Scientific, Inc.). The empty vector was used as a negative control. All primary antibodies E-cadherin (cat no. SAB4503751), N-cadherin (cat no. SAB5500023), vimentin (cat no. SAB4503083) and goat anti-rabbit IgG secondary antibody (A0545) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The anti-human siRNA specifically targeting CCAT1 (synthesized by Thermo Fisher Scientific, Inc.) was used: 5'-CGGCAGGCATTAGAGATGAACAGCA-3'; and the scramble siRNA was used: 5'-CCUACGCCACCAAUUCGU-3'. Cells were transfected using a Lipofectamine 3000 kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Cells were transfected for 48 h prior to their use in CCAT1 expression analyses.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA from the collected tissues was extracted using TRIzol reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol, and cDNAs from all samples were synthesized by PrimeScript RT Master Mix kit (Kusatsu, Japan) and incubated for 30 min at 37°C, 5 sec at 85°C, and then maintained at 4°C. The expression of CCAT1 was quantified using a Quantifast SYBR-Green RT-qPCR kit (Qiagen, GmbH, Hilden, Germany). The primer sequences used were as follows: CCAT1 forward, 5'-CATTGGGAAAGGTGCCGAGA-3'; CCAT1 reverse, 5'-ACGCTTAGCCATACAGAGCC-3'; β -actin forward, 5'-AGCGAGCATCCCCAAAGTT-3'; and β -actin reverse, 5'-GGGCACGAAGGCTCATCATT-3'. PCR was then performed at 94°C for 2 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 30 sec. Each experiment was repeated independently three times. Gene expression levels were analyzed using the 2^{- $\Delta\Delta C_q$} method (9).

Transwell assay. For the migration and invasion assays, 48 h after transfection, 3x10⁴ cells in serum-free RPMI-1640 medium were placed into the upper chamber of each Transwell

Table I. The associations between CCAT1 expression and clinicopathological parameters.

Characteristics	No. of patients	CCAT1 expression, n		P-value
		Low	High	
Age (years)				0.231
≤60	80	40	40	
>60	32	20	12	
Sex				0.860
Male	57	31	26	
Female	55	29	26	
EGFR				0.011
Negative	74	46	28	
Positive	38	14	24	
pT				0.539
1	47	28	19	
2	58	29	29	
3	6	3	3	
4	1	0	1	
pN				0.003
0	63	43	20	
1	11	4	7	
2	35	11	24	
3	3	2	1	
pTNM stage				0.003
I	53	36	17	
II	21	12	9	
III	38	12	26	

Data were analyzed using the χ^2 test. CCAT1, colon cancer-associated transcript 1; EGFR, epidermal growth factor receptor; pT, pathological tumor stage; pN, pathological node stage; pTNM stage, pathological Tumor-Node-Metastasis stage.

insert (8.0 μ m; Millipore, Merck KGaA, Darmstadt, Germany). The chambers were pre-coated with Matrigel (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for the invasion assays. The chambers were then incubated for 24 h at 37°C in a 5% CO₂ incubator, with RPMI-1640 containing 10% FBS in the bottom chambers. Following incubation, cells on the upper surface were scraped off with a cotton swab and washed away with PBS (Invitrogen; Thermo Fisher Scientific, Inc.) and the invaded cells on the lower surface were fixed in 100% precooled methanol for 10 min and stained with 0.5% crystal violet for 2 h. Finally, the number of invaded cells relative to the control group was calculated by counting three fields per membrane under an inverted biological microscope (magnification, x200). Experiments were independently repeated in triplicate.

Western blot analysis. Cells were lysed in RIPA buffer (Sigma-Aldrich; Merck KGaA) supplemented with a protease inhibitor (Roche, Basel, Switzerland) and a phosphatase inhibitor (Roche). Protein concentration was measured using a

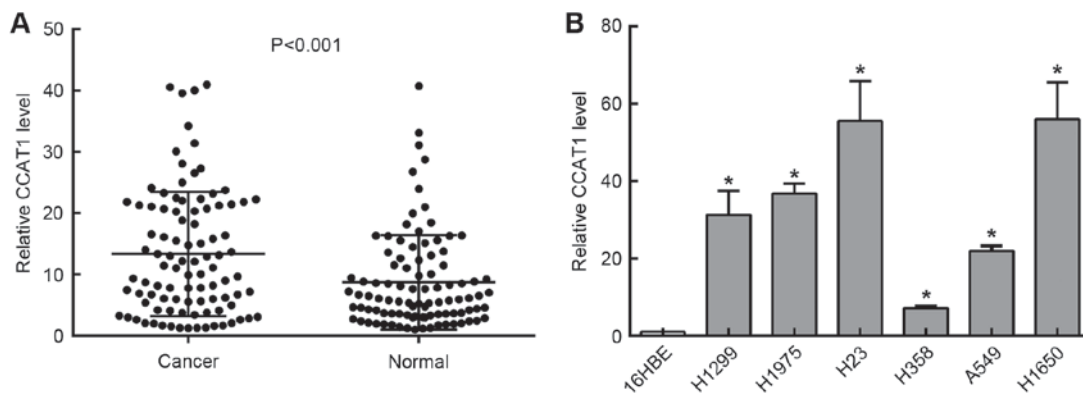


Figure 1. CCAT1 expression in lung adenocarcinoma tissues and cell lines. (A) Expression levels of CCAT1 in human lung adenocarcinoma tissues and corresponding adjacent tissues determined by RT-qPCR (n=96, analyzed by Student's t-test). The mean expression level of CCAT1 in lung adenocarcinoma tissues was significantly higher than that in corresponding non-malignant lung tissues. β -actin was used as an internal control. (B) Expression level of CCAT1 in lung adenocarcinoma cell lines determined by RT-qPCR. The mean expression level of CCAT1 in lung adenocarcinoma cells was significantly higher than that in the human bronchial epithelial cell line 16HBE. β -actin was used as an internal control. * $P < 0.01$ vs. 16HBE (analyzed by one-way ANOVA). CCAT1, colon cancer-associated transcript 1; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

BCA protein Assay kit (Thermo Fisher Scientific, Inc.). Total protein lysates (25 μ g) were fractionated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and were electrophoretically transferred to polyvinylidene difluoride membranes (Roche). Enhanced chemiluminescence (Tiangen Biotech Co., Ltd., Beijing, China) was used to visualize the antibody binding. Equal protein loading was controlled by using a mouse anti- β -actin monoclonal antibody. The membranes were blocked with 10% non-fat milk in Tris-buffered saline Tween (TBST) for 2 h at room temperature, washed and then probed with the primary antibodies (dilution 1:100) overnight at 4°C, followed by incubation with the goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (dilution 1:2,000, TBST dilution) for 2 h at room temperature. The membranes were then washed three times for 15 min with PBST. Proteins were detected using a Molecular Imager ChemiDoc XRS System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The integrated densities of the bands were quantified by ImageJ version software (v1.7.0, National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Statistical analysis was performed and presented using GraphPad Prism version 6.0 (GraphPad Software Inc., La Jolla, CA, USA). The data obtained from *in vitro* assays were analyzed by paired Student's t-test, χ^2 test, Wilcoxon rank-sum (Mann-Whitney) test or one-way ANOVA followed by Tukey's multiple comparison tests, as appropriate. All tests performed were two-sided. $P < 0.05$ was considered to indicate a statistically significant difference.

lncRNA CCAT1 is upregulated in human lung adenocarcinoma tissues and cell lines. The expression levels of CCAT1 were analyzed in cancer tissues (n=96) and adjacent normal tissues from patients with lung adenocarcinoma by RT-qPCR. The results indicated that CCAT1 expression, relative to β -actin, was significantly higher in lung adenocarcinoma tissues than adjacent normal tissues ($P < 0.001$; Fig. 1A). To further investigate the associations between clinicopathological features and CCAT1 expression levels, the mean CCAT1 expression level among all lung adenocarcinoma tissues (mean, 13.33)

was used as the cutoff value. All 96 patients were divided into a low-CCAT1 expression group (n=60) and a high-CCAT1 expression group (n=56). CCAT1 expression was significantly associated with EGFR expression ($P = 0.011$), lymphatic metastasis ($P = 0.003$) and TNM stage ($P = 0.003$), whereas no significant association with age or sex were observed (Table I). The expression of CCAT1 was then detected in lung adenocarcinoma cell lines (H358, H23, H1975, H1650, H1299 and A549) and a normal human bronchial epithelial cell line (16HBE). The RT-qPCR assay indicated that CCAT1 was higher in all lung adenocarcinoma cell lines than in the normal lung cell line ($P < 0.01$; Fig. 1B). Based on CCAT1 expression patterns, the H358 and H1650 cell lines were selected to detect the effect of lncRNA CCAT1 in *in vitro* experiments.

CCAT1 regulates the migration and invasion of lung adenocarcinoma cell lines *in vitro*. To explore the biological functions of CCAT1 in the cell migration and invasion of lung adenocarcinoma, CCAT1 expression was upregulated in H358 cells by transfection with a pcDNA3.1-CCAT1 plasmid, and downregulated in H1650 cells by interfering with siRNA. The transfection efficiency was determined by RT-qPCR (Fig. 2A). A Transwell assay indicated that upregulation of CCAT1 promoted the migration of H358 cells, while suppression of CCAT1 inhibited the migration of H1650 cells, ($P < 0.01$; Fig. 2B). Furthermore, upregulated CCAT1 increased H358 cell invasion, while knockdown of CCAT1 suppressed H1650 cell invasion ($P < 0.01$; Fig. 2C). The results suggested that aberrant expression of CCAT1 acts to regulate lung adenocarcinoma cell metastasis.

lncRNA CCAT1 influences the expression of EMT-associated markers in lung adenocarcinoma. To explore whether aberrant CCAT1 expression levels were involved in the process of EMT, the mesenchymal markers N-cadherin and vimentin, and the epithelial marker E-cadherin were investigated by western blotting. Overexpression of CCAT1 in H358 cells resulted in upregulation of N-cadherin and vimentin protein expression and downregulation of E-cadherin protein; while knockdown of CCAT1 expression in H1650 cells resulted in downregulation

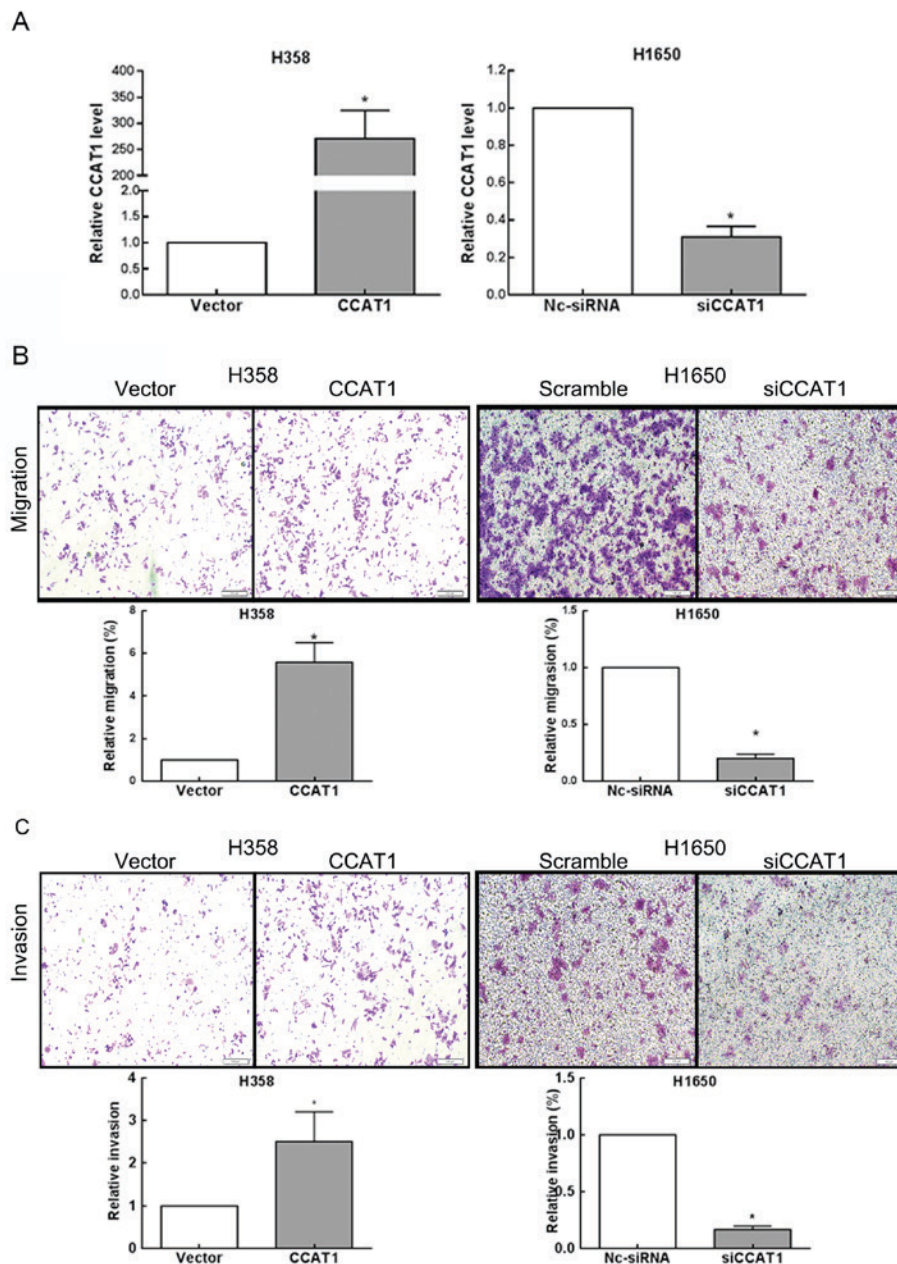


Figure 2. CCAT1 regulates cell migration and invasion *in vitro*. (A) CCAT1 expression levels were determined using reverse transcription-quantitative polymerase chain reaction in H358 and H1650 cells transfected with pcDNA3.1-CCAT1 or an empty vector. β -actin was used as an internal control. Corresponding P-values analyzed by Mann-Whitney U-test are indicated. (B) Representative images and quantification of Transwell migration assays for H358 and H1650 cells indicated that CCAT1 promotes cells migration *in vitro* (scale bar, 50 μ m). * $P < 0.01$. Corresponding P-values analyzed by Mann-Whitney U-test are indicated. (C) Representative images (up) and quantification (down) of Transwell invasion assays for H358 and H1650 cells demonstrated that CCAT1 promotes cells invasion *in vitro* (scale bar, 50 μ m). * $P < 0.01$. Corresponding P-values analyzed by Mann-Whitney U-test are indicated. CCAT1, colon cancer-associated transcript 1; siCCAT1, small interfering RNA against colon cancer-associated transcript 1; Nc-siRNA, noncoding small interfering RNA.

of N-cadherin and vimentin protein expression and upregulation of E-cadherin protein ($P < 0.01$; Fig. 3). This indicates that CCAT1 may contribute to the regulation of EMT-associated marker expression in lung adenocarcinoma cell lines.

Discussion

In the present study, the expression of the lncRNA CCAT1 and its biological effect on tumor cell metastasis in lung adenocarcinoma were detected and investigated, and it was confirmed

that CCAT1 is upregulated in lung adenocarcinoma. Clinically, high expression levels of CCAT1 were associated with EGFR expression, lymphatic metastasis and tumor stage. The clinical results indicated that CCAT1 may be involved in the development of lung adenocarcinoma. Furthermore, upregulation of CCAT1 promoted the migration and invasion of lung adenocarcinoma cell lines, and downregulation of CCAT1 inhibited cell migration and invasion *in vitro*. Additionally, the results demonstrated that CCAT1 may induce EMT to promote cell metastasis in lung adenocarcinoma.

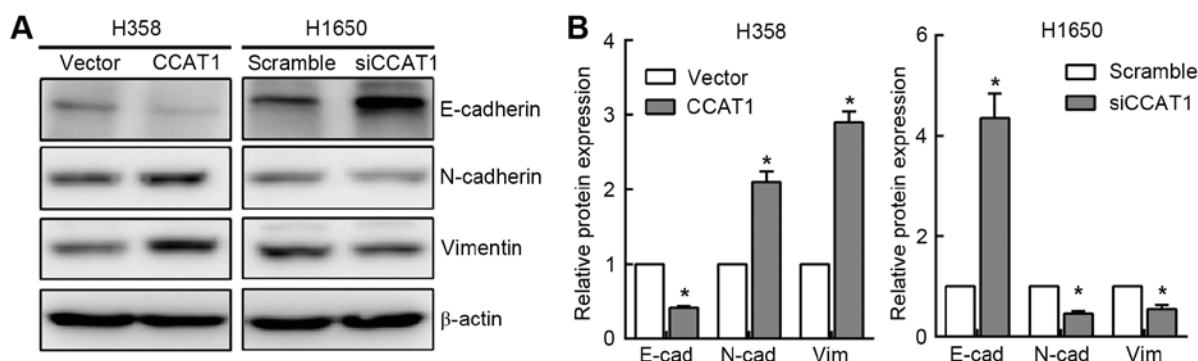


Figure 3. CCAT1 regulates the expression levels of epithelial-to-mesenchymal transition-associated markers. (A) N-cadherin, vimentin and E-cadherin protein expression levels in lung adenocarcinoma cells were analyzed by western blotting. CCAT1 overexpression promoted the expression of N-cadherin and vimentin, while suppressing E-cadherin expression *in vitro*. β -actin was used as a loading control. (B) Average values of integrated optical density of the bands were calculated by analyzing the results of five repeated experiments. Data are presented as the mean \pm standard error of the mean. * $P < 0.01$. Corresponding P-values determined by Mann-Whitney U-test are indicated. CCAT1, colon cancer-associated transcript 1; siCCAT1, small interfering RNA against colon cancer-associated transcript 1; E-cad, E-cadherin, N-cad, N-cadherin; vim, vimentin.

lncRNAs have been reported to serve important regulatory roles in the biology of human malignancy (10). Specifically for lung adenocarcinoma, it has already been reported that lncRNAs contribute to the malignant phenotype of tumor cells (11-16). The lncRNA CCAT1 (NR_108049.1) was initially identified as a strongly upregulated noncoding transcript (2,795 bases in humans) in colon cancer, and high CCAT1 expression is associated with metastasis in colon cancer (17). Recently, Chen *et al* (18) identified that CCAT1 is responsible for docetaxel resistance in lung adenocarcinoma cells. The present study demonstrated that CCAT1 was significantly increased in the patient-derived lung adenocarcinoma tissues compared with the corresponding normal tissues, with the expression of CCAT1 in lung cancer cell lines further confirming the results. Additionally, due to the positive association between CCAT1 and high tumor burden (CCAT1 expression was significantly associated with lymphatic metastasis and TNM stage), cell migration and invasion assays were performed. The results suggested that upregulation of CCAT1 induced cell migration and invasion, while downregulation of CCAT1 inhibited cell metastasis, which meant that CCAT1 could promote the malignant phenotype of lung adenocarcinoma, with the results being consistent with those of Luo *et al* (19). Mechanistically, CCAT1 functions as an oncogene in lung adenocarcinoma through the upregulation of EMT-associated proteins.

EGFR status is a critical factor for the selection of therapeutic strategy in patients with lung adenocarcinoma. In the present study, the results indicated that CCAT1 expression was associated with EGFR status; as EGFR-mutated NSCLC patients can benefit from EGFR-tyrosine kinase inhibitor (TKI) therapies, genes associated with EGFR status may also act as pivotal therapeutic targets. The mechanisms underlying this relationship remain unclear; therefore future studies should focus on the role of CCAT1 in EGFR mutation, and whether it is involved in the development of resistance to EGFR-TKIs in lung cancer. Previous studies have implicated the activation of EMT in resistance to EGFR-TKIs; activation of EMT can suppress E-cadherin and directly reduce sensitivity to EGFR-TKIs (20,21). As a secondary mutation, T790M is the most common mechanism

for acquisition of EGFR-TKI resistance (in 50-60% of cases) (22). Since CCAT1 is an EMT-promoting lncRNA, CCAT1 may also act as a promotion molecule overcoming EGFR-TKI resistance.

Previous studies have demonstrated that CCAT1 can interact with MYC enhancer in colorectal and prostate cancer and, in turn, regulate long-range chromatin interactions at the MYC locus (4), acting as an oncogene sponge in human gallbladder cancer and hepatocellular carcinoma, partly by repressing miRNAs (8,23). CCAT1 can also promote the acquisition of chemoresistance and EMT phenotypes in docetaxel-resistant lung adenocarcinoma cells by competitively binding with let-7c (18). However, as the molecular mechanism of a particular lncRNA may differ in different tumor microenvironments, histological subtypes and tumor stages, CCAT1 may regulate lung adenocarcinoma cell metastasis and EMT phenotypes through different potential targets, and the specific target genes controlled by CCAT1 in lung adenocarcinoma require further studies for clarification.

Taken together, the findings of the present study indicate that aberrant expression of CCAT1 is associated with EGFR status, tumor metastasis and tumor stage in lung adenocarcinoma. In addition, overexpression of CCAT1 promotes metastasis via the induction of EMT *in vitro*. Further research may identify a way in which the pathway linking CCAT1 and EMT may be exploited as a therapeutic approach for the treatment of lung adenocarcinoma.

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Availability of data and materials

The datasets generated in the present study are available on reasonable request from the corresponding author.

Authors' contributions

HL participated in the design of the study and performed the statistical analysis. XZ, WC and HY conceived the study, participated in its design and execution, and helped to draft the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Informed consent was obtained from each patient, and the study was approved by the Ethics Committee of The Third Affiliated Hospital of Wenzhou Medical University (Wenzhou, China).

Consent for publication

Consent for publication was obtained from the study participants.

Competing interests

The authors declare that they have no competing interests.

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