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Original Article

The long non-coding RNA CCAT1 promotes erlotinib resistance in cholangiocarcinoma by inducing epithelial-mesenchymal transition via the miR-181a-5p/ROCK2 axis

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Abstract: Cholangiocarcinoma (CCA) is a common malignancy of the digestive system, and its treatment is greatly challenged by rising chemoresistance. Long non-coding RNAs (lncRNAs) have been shown to play critical roles in the development of drug resistance in tumors. However, the role of the lncRNA CCAT1 in erlotinib resistance in CCA remains unclear. In this investigation, we identified CCAT1 as a pivotal factor contributing to erlotinib resistance in CCA. Furthermore, we uncovered that lncRNA CCAT1 modulated epithelial-mesenchymal transition (EMT) through Rho-associated coiled-coil-forming protein kinase 2 (ROCK2), thereby conferring erlotinib resistance upon CCA cells. Mechanistically, we demonstrated that miR-181a-5p interacted with CCAT1 to modulate the expression of ROCK2. Collectively, these findings shed light on the significant role of CCAT1 in the development of erlotinib resistance in CCA. The functional suppression of CCAT1 holds promise in enhancing the sensitivity to erlotinib by reversing EMT through the miR-181a-5p/ROCK2 signaling pathway. These findings provide valuable insights into the mechanisms underlying erlotinib resistance in CCA and the potential strategies for its treatment.

Keywords: Cholangiocarcinoma, erlotinib resistance, lncRNA CCAT1, ROCK2, EMT

Introduction

Cholangiocarcinoma (CCA) is the third most common malignant tumor of the digestive system, and its morbidity rate is rising globally every year [1]. Early-stage CCA can be radically treated by surgery, but most patients are diagnosed in the middle or advanced stage of the disease and can only receive drug treatment [2]. Currently, the five-year survival rate of CCA patients receiving traditional chemotherapy is only 9%. Targeted therapy is the latest breakthrough in the field of oncology, but only a few targeted drugs are available for CCA, most of which are primarily used in clinical trials [3].

Erlotinib is currently the first-line drug to treat bile duct cancer with great efficacy [4, 5]. However, independent clinical trials showed that erlotinib failed to satisfactorily treat CCA, mainly due to resistance to the drug [6]. The key to unlocking targeted therapy for CCA lies in overcoming the problem of erlotinib resistance. Therefore, we must urgently investigate the

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mechanisms of erlotinib resistance in CCA and explore molecular targets to enhance the sensitivity of this drug.

Mesenchymal-epithelial transition (MET) and epithelial-mesenchymal transition (EMT) play essential roles in chemoresistance in various tumors [7, 8]. EMT is the process through which epithelial cells gain mesenchymal features in terms of morphology, function, structure, migration, and adhesion, while MET is the inverse process of EMT. The two processes can transform into each other, collectively termed EMT-MET transition [9]. EMT is mainly characterized by the downregulation of the molecular marker E-cadherin in epithelial cells and the upregulation of the molecular marker vimentin in mesenchymal stromal cells [10]. Chemotherapeutic agents can enhance the malignancy of chemoresistant tumor cells by inducing the EMT phenotype [11]. The development of EMT has been shown to be closely related to erlotinib resistance [12].

Rho-associated coiled-coil-forming protein kinase 2 (ROCK2) was shown to promote EMT by inhibiting the transcription of cytokeratins, E-cadherin, macrophage function-associated antigen-1, bridging granule proteins, and mucins [13]. ROCK2 is highly expressed in various tumors, such as breast cancer, liver cancer, lung cancer, and prostate cancer [14-17]. ROCK2 has also been shown to modulate the EMT-MET transition in cells and to play an essential role in chemotherapy. Ye et al. confirmed that the downregulation of ROCK2 suppressed EMT and enhanced chemosensitivity in lung cancer [18]. Zhou et al. proved that ROCK2 promoted EMT, resulting in the development of gemcitabine resistance in patients with pancreatic cancer [19]. ROCK2 was also reported to be associated with the malignancy of CCA [20]. However, the effects of ROCK2-mediated EMT on erlotinib resistance in CCA and its underlying mechanism are still unclear.

Whole-genome sequencing has revealed that only 2% of the genome can be translated into proteins, with most transcripts being non-coding RNAs [21]. Long non-coding RNAs (lncRNAs) are a family of RNAs over 200 nucleotides long that are involved in RNA transcription, protein translation, DNA replication, and post-translational modification [21]. lncRNAs have been demonstrated to be involved in various cellular activities, including cell proliferation, metabolism, invasion, metastasis, apoptosis, drug resistance, and the maintenance of stemness [22]. lncRNAs can also regulate tumor development by modulating tumor drug resistance [23]. The lncRNA ACTA2-AS1 induced cisplatin resistance in patients with ovarian cancer by binding to miR-378a-3p [24]. lncRNA-MIR15-5HG regulated the expression of annexin A2 and established drug resistance in colorectal cancer [25]. On the other hand, Shi et al. reported that lncRNA-DLA1 reversed chemoresistance in breast cancer cells by inhibiting the degradation of cyclin D1 [26]. Overall, these studies suggest that lncRNAs can be used as potential targets for modulating chemoresistance in tumors.

In this study, we used multi-omics analysis to identify the lncRNA CCAT1 as a key mediator of erlotinib resistance in CCA. Furthermore, we examined the impact of CCAT1 on erlotinib resistance and explored the therapeutic significance of erlotinib resistance in CCA patients. We discovered that the lncRNA CCAT1 promoted erlotinib resistance in CCA by inducing EMT via the miR-181a-5p/ROCK2 axis.

Methods

Cell lines and reagents

The human bile duct cancer cell lines HCC-9810 and RBE were supplied by Shanghai Anwei Biotechnology Co., Ltd. (Shanghai, China) and verified by short tandem repeat profiling. RBE cells were cultivated in Dulbecco's Modified Eagle Medium (Gibco, Carlsbad, CA) supplemented with 100 U/mL penicillin/streptomycin and 10% fetal bovine serum (Invitrogen), whereas HCC-9810 cells were cultured in Roswell Park Memorial Institute 1640 medium supplemented with 100 U/mL penicillin/streptomycin and 10% fetal bovine serum. Both cell lines were maintained in an incubator at 37°C with 5% CO_2.

Cell transfection

sh-NC lentivirus, sh-ROCK2 lentivirus and sh-CCAT1 lentivirus were purchased from Focus Bioscience (Nanchang, China). Erlotinib-resistant (ER) CCA cells were transfected with the lentiviral vectors, cultured for 16 h, and further cultured for an additional 48 h in fresh medi-

um. The expression of ROCK2 and CCAT1 was evaluated by quantitative reverse transcription-PCR (qRT-PCR). To create stably transfected cell lines, the infected CCA cells were co-cultured with 1 μg/mL puromycin until every cell in the control group died. The successfully constructed stable cell lines were used in the subsequent experiments. The sequences of shRNA targeting CCAT1 and ROCK2 were listed as follows: CCAT1-shRNA1: 5'-CCATTCCATTCATTTCTCTTT-3'; CCAT1-shRNA2: 5'-AAGCAGGCAGAAAGCCGTATC-3'. ROCK2-shRNA1: 5'-GCACAGTTTGAGAAGCAGCTA-3'; ROCK2-shRNA2: 5'-GCCTTGCATATTGGTCTGGAT-3'.

RNA extraction and qRT-PCR

Total RNA was extracted from cultivated CCA cells using TRIZOL reagent (Invitrogen, 155-96026), quantified on the Evolution 350 ultraviolet-visible spectrophotometer (Thermo), reverse-transcribed using the PrimeScript kit with gDNA Eraser (Takara, RR047A), and subjected to qRT-PCR using the TB Green® PreMix Ex Taq Quantity (Tli RNaseH Plus) kit (Takara, RR420A). The expression levels of miR-181a-5p, ROCK2, and CCAT1 were determined using the 2-ΔΔCt method and normalized to their respective controls. The controls used were GAPDH (for CCAT1 and ROCK2) and U6 (for miR-181a-5p). All measurements were performed in triplicate. Primers were acquired from Genomeditech (Guangzhou, China), and their sequences are given below: GAPDH Forward Primer: 5'-GGCTACAGCAATGGCTACC-3'; GAPDH Reverse Primer: 5'-GATGCCGCTGAGAGTGAC-3'. CCAT1 Forward Primer: 5'-ATCGTGGGGCTTCTCATGTTT-3'; CCAT1 Reverse Primer: 5'-TTAGCATGCCCCAACTTCACTTT-3'. ROCK2 Forward Primer: 5'-GGCCGTCAGAGGAAGCTGGAGG-3'; ROCK2 Reverse Primer: 5'-TTAGCTTGGCTTGTTTGGAGCA-3'. The miR-181a-5p-specific forward primer was AACATTCAACGCTGTCGGTGAGT, and U6 served as a standardization control.

Western blotting

Total protein was extracted from CCA cells using radioimmunoprecipitation assay lysis buffer, quantified using the bicinchoninic acid assay, mixed with protein loading buffer, and boiled for 10 min. Proteins were resolved by electrophoresis on 7.5% or 10% sodium dodecyl sulfate-polyacrylamide gels and transferred to a 0.22-μm polyvinylidene difluoride membrane. The membrane was blocked with 5% skim milk for 60 min at room temperature (RT), then incubated with the appropriate primary antibodies at 4°C overnight. After washing three times for 10 min each with 1× Tris-buffered saline with Tween 20, the membrane was incubated with the appropriate secondary antibodies for 1 h at RT, washed again three times for 10 min each with 1× Tris-buffered saline with Tween 20, and exposed to enhanced chemiluminescence reagents for imaging. ImageJ was used to statistically analyze the data.

Cell viability assay

CCA cells in their logarithmic growth phase were seeded in a 96-well plate (3×10^3 cells/100 μL), allowed to adhere, and incubated for 72 h with culture media containing different doses of erlotinib. After this, cells were incubated for 2 h with cell counting reagent (10 μL/100 μL; GLPBIo, GK10001). Absorbance was determined at 450 nm using a microplate reader. Cell viability (%) was calculated using the following formula: Cell viability (%) = (ODtreatment - ODblank)/(ODcontrol - ODblank) × 100%, where OD denotes the optical density averaged over three wells.

Colony formation assay

CCA cells were seeded in a 6-well plate (1500 cells/well), cultured for 2 weeks in a humidified atmosphere with 5% CO_2, stained for 15 min with 0.1% crystal violet, and washed twice with phosphate-buffered saline (PBS). Allow the wells to air dry, and then visualize and count the colonies.

Cell apoptosis assay

Transfected ER CCA cells were treated with 5 μM erlotinib for 3 days and double-stained for apoptosis using the Annexin V FITC/PI apoptosis detection kit (BD Biosciences) according to the manufacturer's instructions. The apoptosis rate was measured using a flow cytometer (BD Biosciences, USA).

Terminal deoxynucleotidyl transferase dUTP nick-end labeling staining

Following transfection, cells were fixed with 4% paraformaldehyde for 30 min and then exposed

to terminal deoxynucleotidyl transferase dUTP nick-end labeling test reagent (Roche, Basel, Switzerland) for 1 h at 37°C. Nuclei were visualized using 4',6-diamidino-2-phenylindole (Beyotime, Shanghai, China). After washing with PBS, the cells were observed under a fluorescence microscope (Olympus, Tokyo, Japan).

Immunofluorescence

CCA cells that had undergone differentiation were seeded in 24-well plates (1.5×10^4 cells/mL), cultured for 1 day, fixed for 15 min with 4% paraformaldehyde, washed three times with PBS for 3 min each, permeabilized with 0.5% Triton X-100 at RT for 20 min. After another three washs with PBS, the cells were blocked with 500 μL/well of 5% goat serum incubated at RT for 30 min to prevent nonspecific binding. The cells were then incubated overnight at 4°C with antibodies against N- and E-cadherin (Proteintech, 20874-1-AP and 60330-1Ig, respectively), washed three times with PBS containing Tween 20, incubated with fluorescent secondary antibodies for 1 h, stained with 4',6-diamidino-2-phenylindole for 5 min to visualize the nuclei, washed three more times with PBS containing Tween 20, and finally examined under a fluorescence microscope.

RNA immunoprecipitation assays

The Magna RIP RNA binding protein immunoprecipitation kit (Millipore, USA) was used to perform RNA immunoprecipitation in compliance with the manufacturer's recommendations. Ago2 (1:50; Merck Millipore) was employed to facilitate RNA immunoprecipitation. The lysate was incubated with RNase for 1 h at 37°C. The co-precipitated RNA was determined using RT-PCR.

Luciferase reporter assay

Mutant (MUT) and wild-type (WT) CCAT1 and ROCK2 were amplified and cloned into the luciferase reporter vector psiCHECK-2 (Promega, Madison, WI, USA) to obtain MUT-CCAT1, WT-CCAT1, MUT-ROCK2, and WT-ROCK2, which were co-transfected with negative control or miR-181a-5p into cells using Lipofectamine 3000 (Invitrogen, Carlsbad, MA, USA). The luciferase activity was assayed 2 days post-transfection using a dual luciferase reporter gene assay system (Promega, Madison, WI, USA).

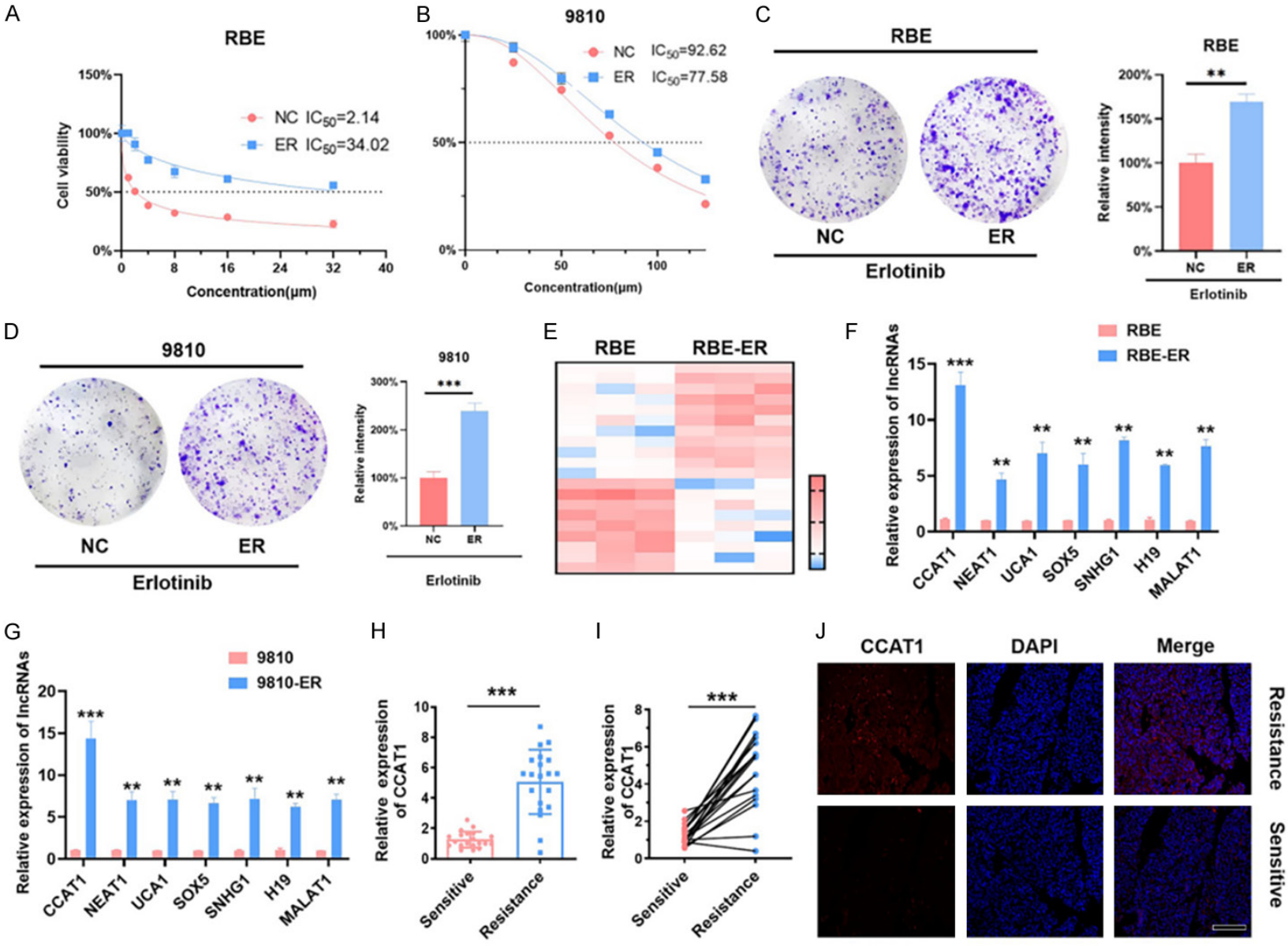
Statistical analyses

The data are presented as the mean ± standard deviation of three independent experiments. Student's t test and one-way analysis of variance were applied to examine the differences between two groups and between multiple groups, respectively. Dunnett's post-hoc test was subsequently used for analysis. All statistical computations were carried out in IBM SPSS 19.0. All the graphics were created using GraphPad Prism 8.0 (GraphPad software). A difference was considered statistically significant if P was less than 0.05.

Results

CCAT1 was highly expressed in ER CCA cells and tissues

To elucidate the molecular mechanism of erlotinib resistance in CCA, we first used the concentration increment method to construct the ER CCA cell lines RBE-ER and HCCC-9810-ER. We measured the half-maximal inhibitory concentrations (IC50) of erlotinib in the parental and resistant strains and performed clone formation assays to assess the degree of resistance among ER strains. The IC50 values of erlotinib in HCCC-9810-ER and RBE-ER cells were significantly higher than those in HCCC-9810 and RBE cells (**Figure 1A**, **1B**). Furthermore, the drug-resistant cells formed a greater number of colonies than the parental cells ($P < 0.05$; **Figure 1C**, **1D**). To identify the lncRNAs mediating erlotinib resistance in CCA, the expressions of lncRNAs in resistant and parental cells were compared using a microarray. The heat map illustrated a notable distinction between the resistant and parental cells (**Figure 1E**). qRT-PCR was applied to confirm the six lncRNAs that exhibited the greatest fold-change (> 5), among which CCAT1 exhibited the highest (**Figure 1F** and **1G**). We then examined CCAT1 expression in erlotinib-resistance and erlotinib-sensitive CCA tissues. qRT-PCR and in situ hybridization analyses revealed that CCAT1 was remarkably highly expressed in erlotinib-resistance CCA tissues compared with erlotinib-sensitive CCA tissues (**Figure 1H-J**). These results indicate that the lncRNA CCAT1, which was highly expressed in ER CCA cells and tissues, may confer erlotinib resistance upon CCA cells.


A
RBE
Cell viability
150%
100%
50%
0%
0
8
16
24
32
40
Concentration(μm)
NC IC50=2.14
ER IC50=34.02
B
9810
100%
50%
0%
0
50
100
Concentration(μm)
NC IC50=92.62
ER IC50=77.58
C
RBE
NC
ER
Erlotinib
RBE
Relative intensity
200%
150%
100%
50%
0%
**
NC
ER
Erlotinib
D
9810
NC
ER
Erlotinib
9810
Relative intensity
300%
200%
100%
0%
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NC
ER
Erlotinib
E
RBE
RBE-ER
F
RBE
RBE-ER
Relative expression of lncRNAs
15
10
5
0
***
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CCAT1
NEAT1
UCA1
SOX5
SNHG1
H19
MALAT1
G
9810
9810-ER
Relative expression of lncRNAs
20
15
10
5
0
***
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CCAT1
NEAT1
UCA1
SOX5
SNHG1
H19
MALAT1
H
Relative expression of CCAT1
10
8
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4
2
0
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Sensitive
Resistance
I
Relative expression of CCAT1
8
6
4
2
0
***
Sensitive
Resistance
J
CCAT1
DAPI
Merge
Resistance
Sensitive

Figure 1. CCAT1 was highly expressed in ER CCA cells and tissues. (A and B) The IC50 values of erlotinib were measured using cell viability assay in NC and ER RBE (A) and NC and ER 9810 (B) cells. (C and D) Cell proliferation was measured using clone formation assays in NC and ER RBE (C) and NC and ER 9810 (D) cells. (E) The expressions of lncRNAs in resistant and parental cells were detected using a microarray. (F and G) The indicated lncRNAs were detected using qRT-PCR in NC and ER RBE (F) and NC and ER 9810 (G) cells. (H-J) qRT-PCR (H) and In situ hybridization (I and J) analyzes CCAT1 expression in erlotinib-sensitive and erlotinib-resistance CCA tissues.

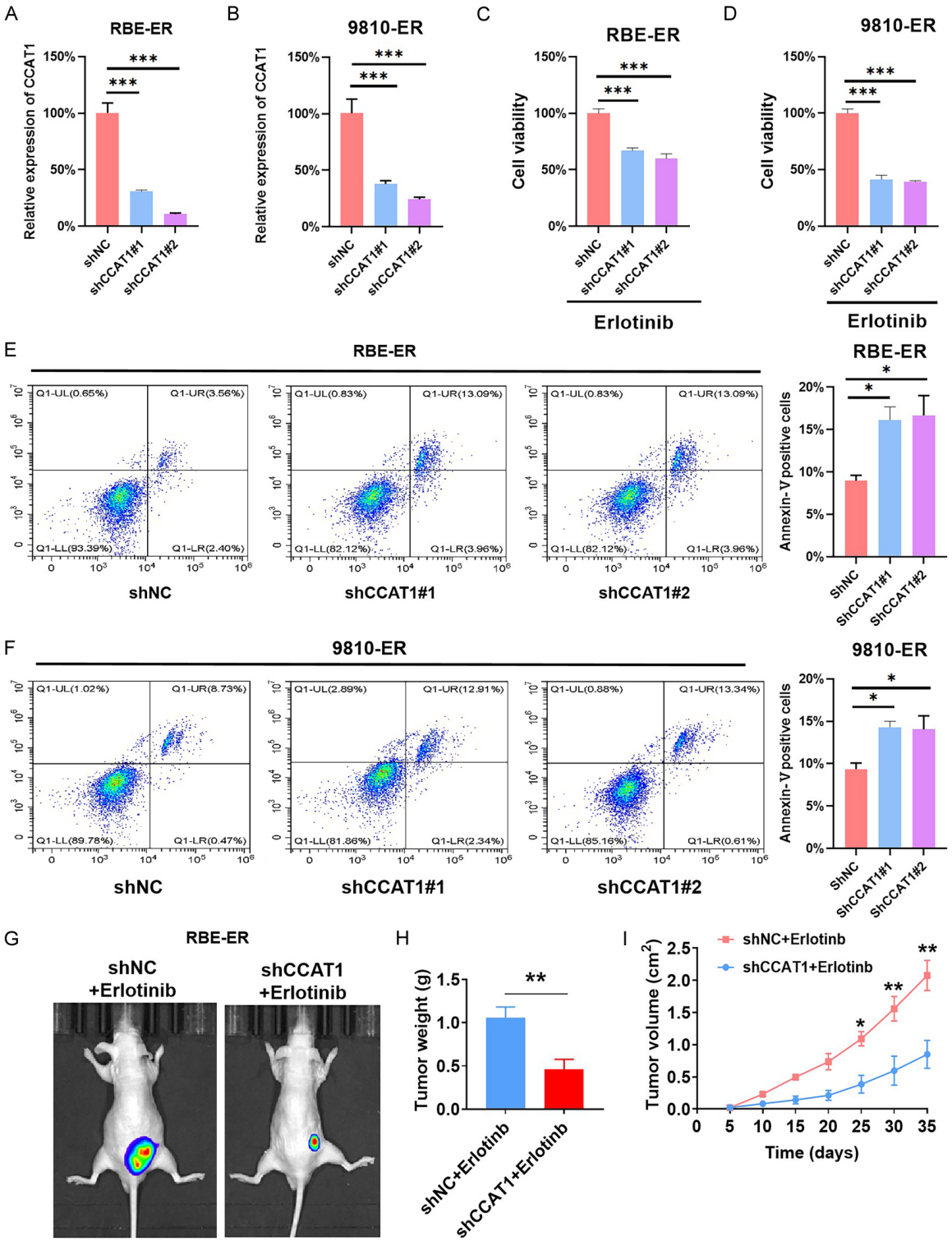


Figure 2. Knockdown of CCAT1 reversed erlotinib resistance in CCA cells. (A and B) qRT-PCR analysis of CCAT1 expression in shCCAT-HCCC-9810-ER (A) and shCCAT-RBE-ER (B) cells. (C and D) cell viability was measured using cell viability assay in shCCAT-HCCC-9810-ER (C) and shCCAT-RBE-ER (D) cells. (E and F) Apoptosis rate was analyzed by cell apoptosis assay in shCCAT-HCCC-9810-ER (E) and shCCAT-RBE-ER (F) cells. (G-I) In vivo tumor formation was examined by subcutaneously injecting shNC-RBE+Erlotinib (left) or shCCAT-RBE+Erlotinib (right) cells into the flank of nude mice. Representative photographs obtained 25 days after inoculation. Tumor weight was measured and the corresponding tumor growth curves were obtained (*$P < 0.05$, **$P < 0.01$).

Knockdown of CCAT1 reversed erlotinib resistance in CCA cells

To examine the functional impact of CCAT1 on erlotinib resistance, we used the lentivirus-mediated short hairpin RNA method to stably knock down CCAT1 in HCCC-9810-ER and RBE-ER cells (**Figure 2A**, **2B**). The suppression of growth and IC50 induced by erlotinib were clearly reversed after CCAT1 was silenced in drug-resistant cells (**Figure 2C**, **2D**). Furthermore, compared with the negative control, the rate of apoptosis was enhanced in CCAT1-knockdown HCCC-9810-ER and RBE-ER cells treated with erlotinib (**Figure 2E**, **2F**). CCAT1 knockdown considerably increased the susceptibility of the RBE-ER xenograft to erlotinib *in vivo* (**Figure 2G-I**). These data suggest that the knockdown of CCAT1 reversed erlotinib resistance in CCA cells.

CCAT1 promoted erlotinib resistance in CCA by inducing EMT

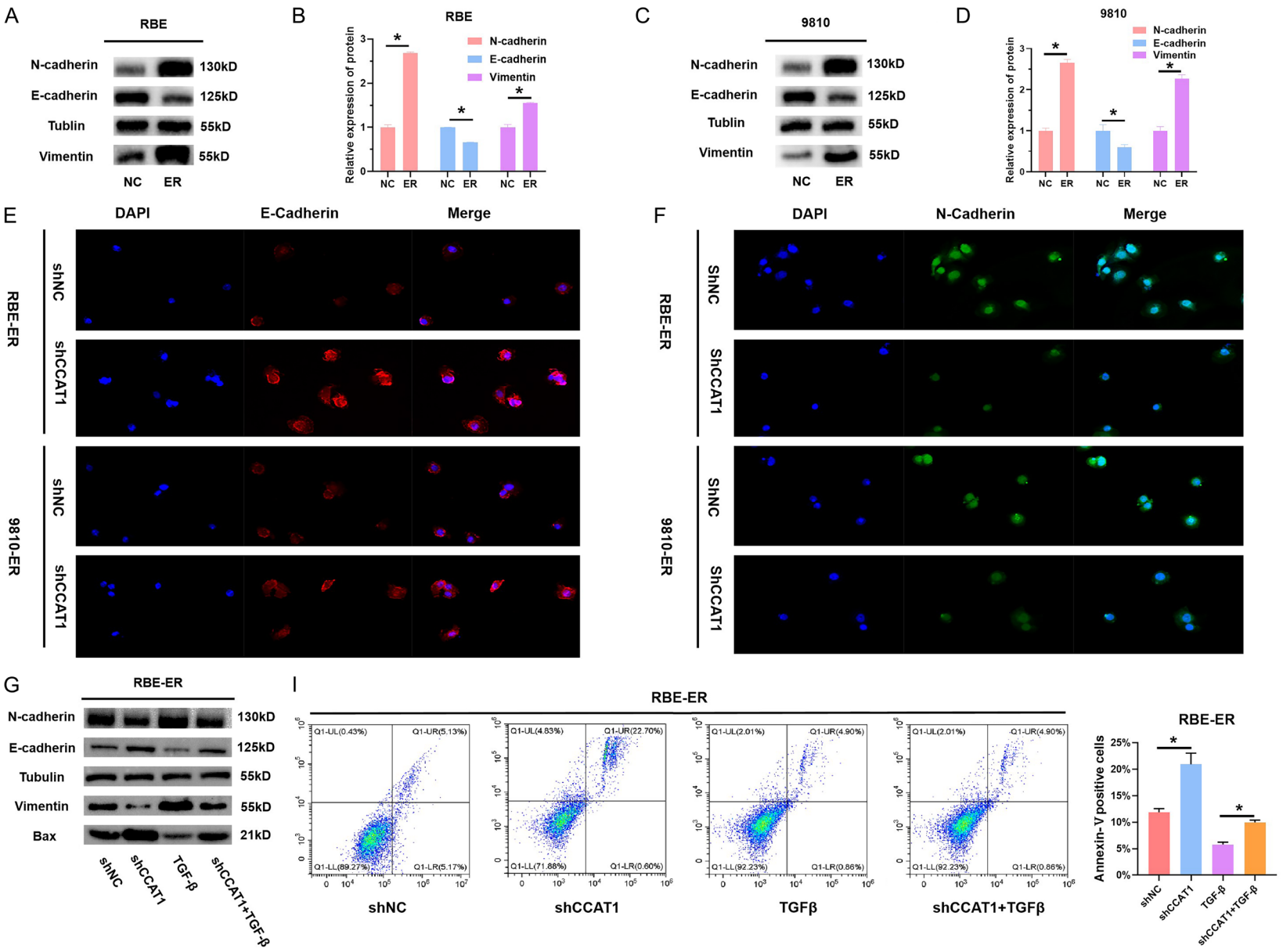
EMT commonly leads to drug resistance in numerous solid tumors. Thus, we hypothesized that CCAT1 might be enhancing drug resistance in CCA cells by controlling EMT. We first examined the expression of EMT-associated proteins in ER and parental cells using Western blotting to elucidate the connection between EMT signaling and CCAT1. The expression of vimentin and N-cadherin was considerably higher while that of E-cadherin was lower in ER cells compared with parental cells (**Figure 3A-D**). Immunofluorescence analysis also indicated the same expression patterns of N- and E-cadherin, suggesting that EMT is a key mediator of erlotinib resistance in CCA cells (**Figure 3E**, **3F**). Furthermore, upregulating CCAT1 expression resulted in decreased E-cadherin and increased N-cadherin expression (Supplementary Figure 1). Moreover, downregulating CCAT1 increased the drug sensitivity of ER CCA cells, but this was reversed when transforming growth factor-β (TGF-β), an EMT activator, was added (**Figure 3G-J**). These findings show that CCAT1 regulated the susceptibility of CCA cells to erlotinib via EMT.

CCAT1 modulated ROCK2-mediated EMT resulting in erlotinib resistance in CCA cells

ROCK2 was shown to be associated with EMT and to regulate CCA cell growth. We first observed ROCK2 expression at the mRNA and protein levels in CCAT1-knockdown CCA cells to confirm whether CCAT1 could control ROCK2 expression. ROCK2 expression was considerably attenuated in CCAT1-knockdown HCCC-9810-ER and RBE-ER cells, as demonstrated by Western blotting and qRT-PCR (**Figure 4A-D**). Subsequently, we silenced ROCK2 expression in ER CCA cells and added an EMT activator to examine changes in the drug sensitivity of these cells. ER CCA cells became more sensitive when ROCK2 expression was attenuated, but this was inhibited when the EMT activator was added (**Figure 4E-H**), suggesting that CCAT1 controls ROCK2-mediated EMT, which conferred erlotinib resistance upon CCA cells.

ROCK2 was a key protein in CCAT1's regulation of EMT leading to erlotinib resistance in CCA

As mentioned above, ROCK2 was identified as a downstream target of CCAT1. To validate whether ROCK2 was a critical protein in CCAT1's regulation of EMT leading to erlotinib resistance in CCA, we overexpressed ROCK2 in CCAT1-knockdown ER CCA cells and observed the levels of EMT markers, ROCK2 protein, cell apoptosis, and proliferation. Downregulating CCAT1 decreased ROCK2 expression, while overexpressing ROCK2 blunted the decrease in N-cadherin expression in CCAT1-knockdown ER CCA cells (**Figure 5A**). Moreover, colony formation and Cell Counting Kit-8 assays revealed that CCAT1 knockdown evidently decreased the proliferation of ER CCA cells, whereas overexpressing ROCK2 inhibited this phenomenon (**Figure 5B**, **5C**). Flow cytometry demonstrated that overexpressing ROCK2 mitigated apopto-


A
RBE
N-cadherin
E-cadherin
Tublin
Vimentin
130kD
125kD
55kD
55kD
NC ER
B
RBE
N-cadherin
E-cadherin
Vimentin
Relative expression of protein
NC ER NC ER NC ER
C
9810
N-cadherin
E-cadherin
Tublin
Vimentin
130kD
125kD
55kD
55kD
NC ER
D
9810
N-cadherin
E-cadherin
Vimentin
Relative expression of protein
NC ER NC ER NC ER
E
DAPI
E-Cadherin
Merge
RBE-ER
shNC
shCCAT1
9810-ER
shNC
shCCAT1
F
DAPI
N-Cadherin
Merge
RBE-ER
ShNC
ShCCAT1
9810-ER
ShNC
ShCCAT1
G
RBE-ER
N-cadherin
E-cadherin
Tubulin
Vimentin
Bax
130kD
125kD
55kD
55kD
21kD
shNC
shCCAT1
TGF-β
shCCAT1+TGF-β
I
RBE-ER
Q1-UL(0.43%)
Q1-UR(5.13%)
Q1-LL(89.27%)
Q1-LR(5.17%)
shNC
Q1-UL(4.83%)
Q1-UR(22.70%)
Q1-LL(71.88%)
Q1-LR(0.60%)
shCCAT1
Q1-UL(2.01%)
Q1-UR(4.90%)
Q1-LL(92.23%)
Q1-LR(0.86%)
TGFβ
Q1-UL(2.01%)
Q1-UR(4.90%)
Q1-LL(92.23%)
Q1-LR(0.86%)
shCCAT1+TGFβ
RBE-ER
Annexin-V positive cells
shNC
shCCAT1
TGF-β
shCCAT1+TGF-β

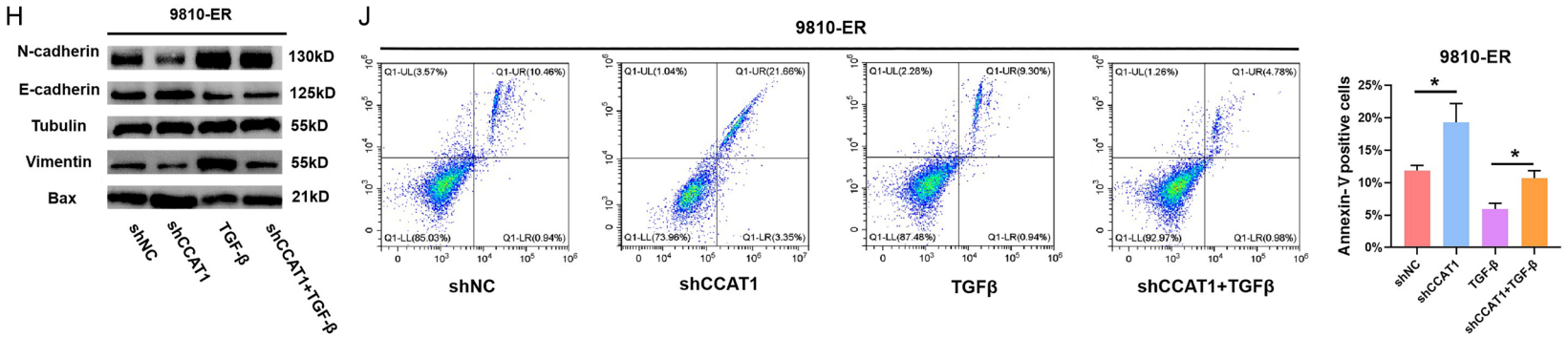


Figure 3. CCAT1 promoted erlotinib resistance in CCA by inducing EMT. (A-D) Western blot analyses were performed to detect the indicated protein expression levels in NC and ER RBE (A and B) and NC and ER 9810 (C and D) cells. (E and F) Immunofluorescence analysis of N- (E) and E-cadherin (F) expression in shCCAT1 CCA cells. (G and H) Western blot analyses were performed to detect the indicated protein expression levels in indicated groups in RBE-ER (G) and 9810-ER (H) cells. (I and J) Apoptosis rate was analyzed by cell apoptosis assay in indicated groups in RBE-ER (I) and 9810-ER (J) cells.

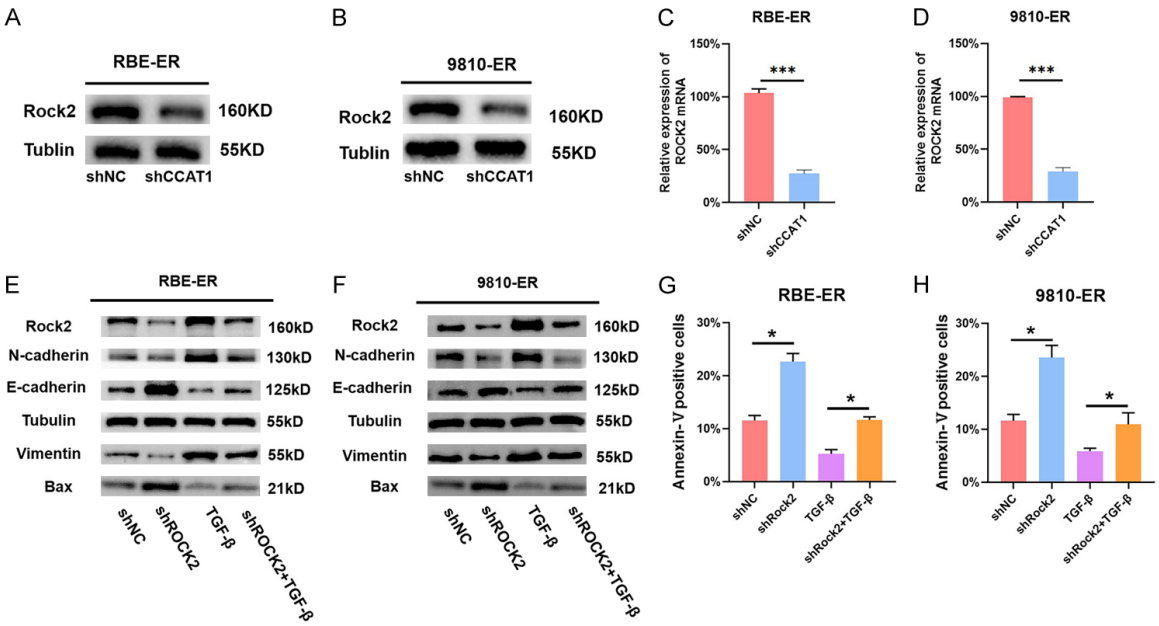


Figure 4. CCAT1 modulated ROCK2-mediated EMT resulting in erlotinib resistance in CCA cells. (A and B) Western blot analyses were performed to detect ROCK2 protein expression level in RBE-ER (A) and 9810-ER (B) cells with shCCAT1. (C and D) qRT-PCR analysis of ROCK2 expression in RBE-ER (C) and 9810-ER (D) cells with shCCAT1. (E and F) Western blot analyses were performed to detect the indicated protein expression levels in indicated groups in RBE-ER (E) and 9810-ER (F) cells. (G and H) Apoptosis rate was analyzed by cell apoptosis assay in indicated groups in RBE-ER (G) and 9810-ER (H) cells.

sis in CCAT1-knockdown CCA cells treated with erlotinib (**Figure 5D**). shROCK2 blunted the increase in N-cadherin expression in overexpressing CCAT1 ER CCA cells, and shROCK2 increased apoptosis in overexpressing CCAT1 CCA cells treated with erlotinib (**Figure 5E-H**). In addition, overexpressing ROCK2 mitigated the susceptibility of the shCCAT1-RBE-ER xenograft to erlotinib *in vivo* (Supplementary Figure 2). These results demonstrated that ROCK2 was a key protein in CCAT1's regulation of EMT leading to erlotinib resistance in CCA.

CCAT1 functions as a competing endogenous RNA for miR-181a-5p to facilitate ROCK2 expression in ER CCA cells

We utilized multiple microRNA bioinformatic databases (Targetscan and Star-base v2.0) to explore the mechanism by which CCAT1 modulated ROCK2. We discovered that ROCK2 and CCAT1 could both bind to miR-181a-5p (**Figure 6A**, **6B**). CCA cells were transfected with a miR-181a-5p mimic to ectopically overexpress miR-181a-5p, which reduced the expression of ROCK2 (**Figure 6C-H**). The interaction between ROCK2 and miR-181a-5p was examined using the luciferase reporter assay. miR-181a-5p evidently reduced the luciferase activity regulated by the WT 3' untranslated region (UTR) of ROCK2 but not that regulated by the MUT 3' UTR of ROCK2 (**Figure 6I**, **6J**).

To understand the connection between ROCK2 and CCAT1, we assessed the expression of ROCK2 in shCCAT1-CCA cells. ROCK2 was downregulated when CCAT1 was knockdown (**Figure 6K**, **6L**). Luciferase reporter assays confirmed the binding between miR-181a-5p and CCAT1 (**Figure 6M**, **6N**), and downregulated miR-181a-5p alleviated the effect of knockdown CCAT1 on ROCK2 (**Figure 6O**, **6P**). Moreover, the increase in CCA cell apoptosis induced by the knockdown of CCAT1 was mitigated by the down-regulation of miR-181a-5p (**Figure 6Q**, **6R**). Overall, these findings demonstrated that the lncRNA CCAT1 induced erlotinib resistance in CCA by inducing EMT via the miR-181a-5p/ROCK2 axis.

Discussion

CCA is a type of malignant tumor of the biliary tract with a heterogeneous and complex tumor

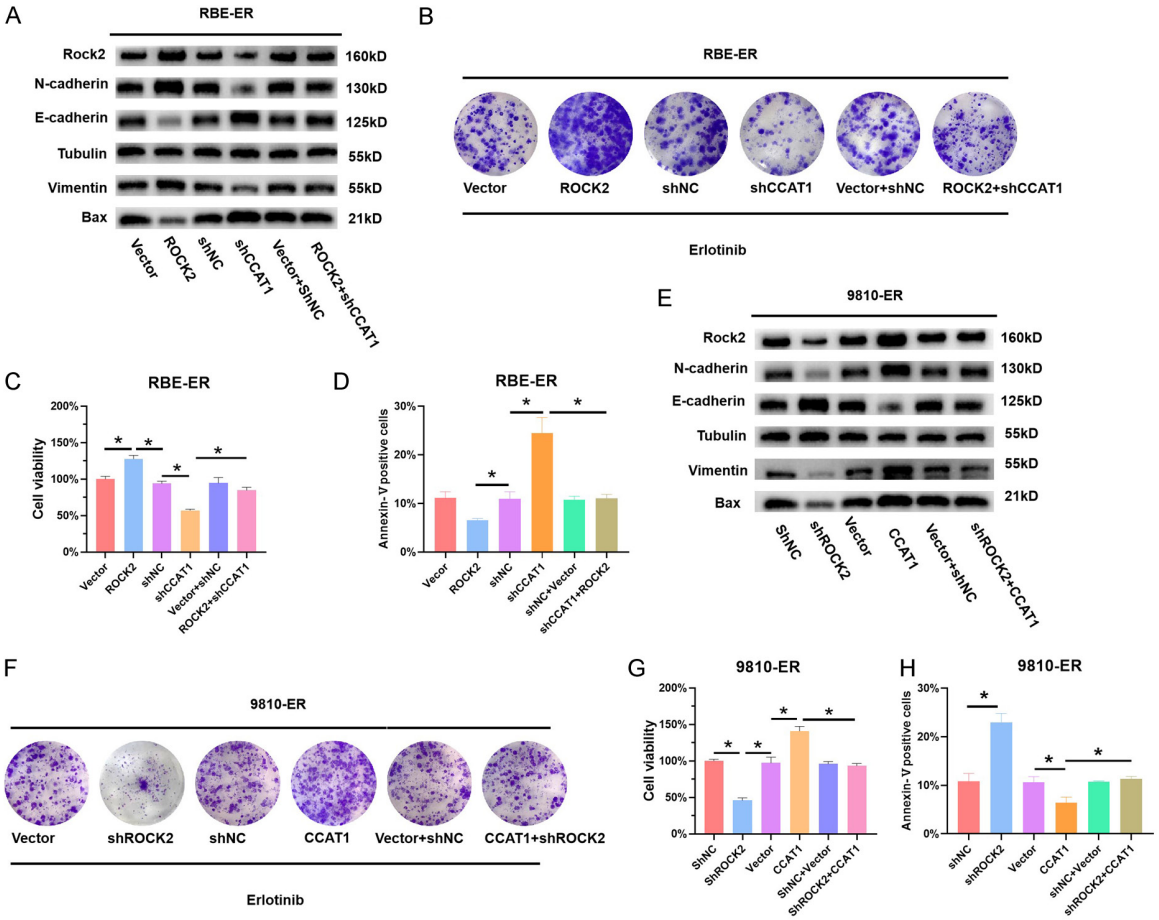
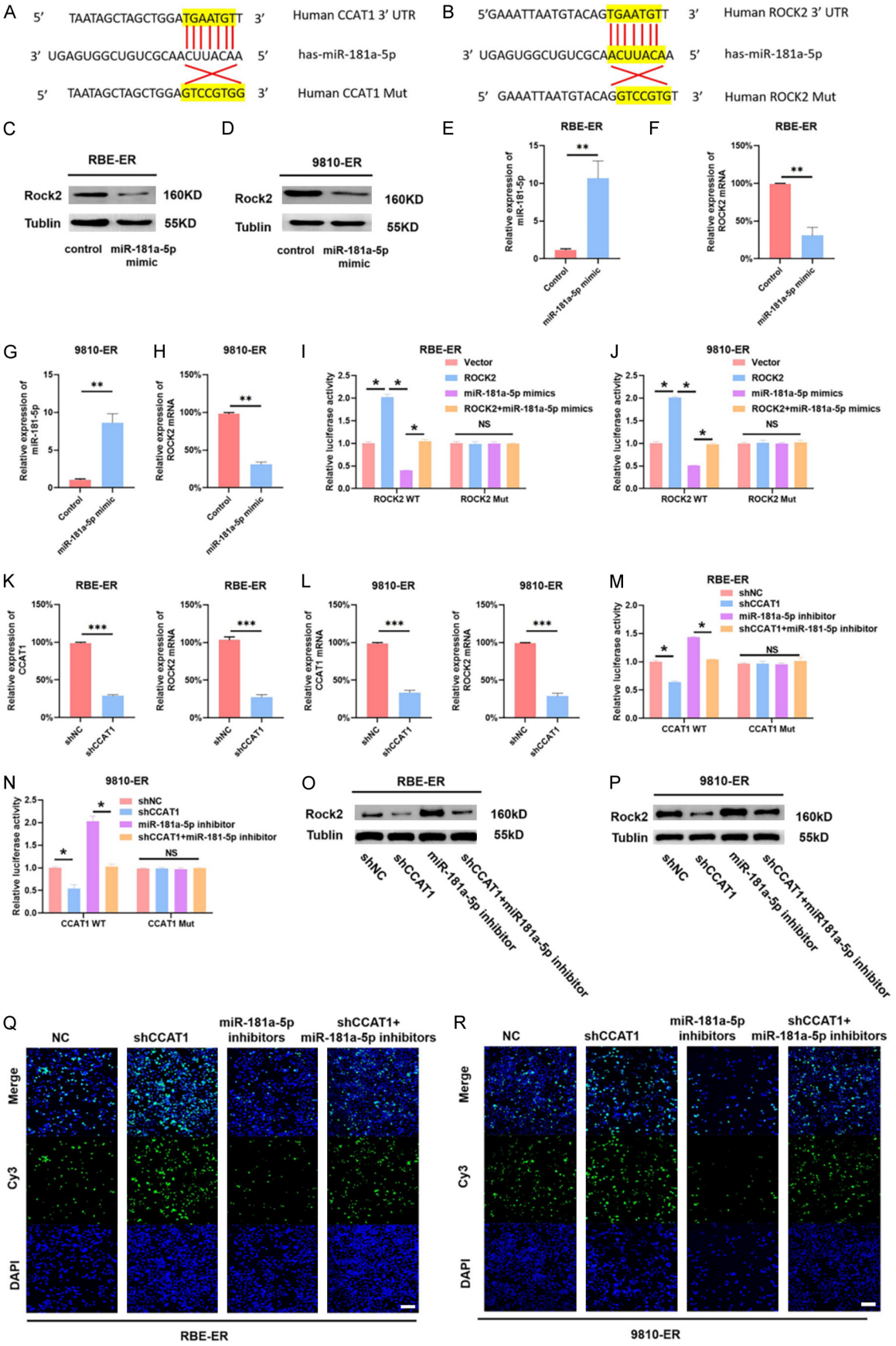


Figure 5. ROCK2 was a key protein in CCAT1's regulation of EMT leading to erlotinib resistance in CCA. (A and E) Western blot analyses were performed to detect the indicated protein expression levels in indicated groups in RBE-ER (A) and 9810-ER (E) cells. (B and F) Cell proliferation was measured using clone formation assays in indicated groups in RBE-ER (B) and 9810-ER (F) cells. (C and G) Cell proliferation was measured using Cell Counting Kit-8 assays in indicated groups in RBE-ER (C) and 9810-ER (G) cells. (D and H) Apoptosis rate was analyzed by cell apoptosis assay in indicated groups in RBE-ER (D) and 9810-ER (H) cells.

microenvironment [1]. It imposes a heavy socioeconomic burden, particularly in low- and middle-income countries where its incidence is high [27]. Due to its late onset and asymptomatic nature, CCA evolves over a decade-long cumulative carcinogenic process. Surgical resection is the first-choice treatment, but due to the rapid progression and high malignancy of CCA, most patients have already lost the window for surgery at the time of diagnosis. Moreover, surgically treated patients still suffer from a high rate of recurrence, which makes radiotherapy an inevitable resort for the clinical treatment of CCA [3]. Erlotinib is currently the first-line drug for effectively treating progressive bile duct cancer [4]. However, erlotinib has not been effective in treating CCA in clinical trials, mainly due to the development of erlotinib resistance in CCA patients. In this study, we have elucidated the mechanism of lncRNA CCAT1-mediated erlotinib resistance in CCA cells.

CCAT1 has been shown to significantly contribute to different stages in the progression of numerous malignancies, such as apoptosis, proliferation, invasion, and mobility [28]. For instance, by blocking miR-181, CCAT1 might upregulate autophagy related 7 and encourage autophagy in hepatocellular carcinoma cells [29]. Tang et al. demonstrated that CCAT1 enhanced stemness in breast cancer cells through Wnt/β-catenin signaling [30]. Our previous study also showed that CCAT1 stimulated invasion and migration in intrahepatic CCA by inhibiting miR-152 [31]. However, the associa-


A
5′ TAATAGCTAGCTGGATGAATGTT 3′ Human CCAT1 3′ UTR
3′ UGAGUGGCUGUCGCAACUUACAA 5′ has-miR-181a-5p
5′ TAATAGCTAGCTGGAGTCCGTGG 3′ Human CCAT1 Mut
B
5′GAAATTAATGTACAGTGAATGTT 3′ Human ROCK2 3′ UTR
3′ UGAGUGGCUGUCGCAACUUACAA 5′ has-miR-181a-5p
5′ GAAATTAATGTACAGGTCCGTGT 3′ Human ROCK2 Mut
C
RBE-ER
Rock2
160KD
Tublin
55KD
control miR-181a-5p mimic
D
9810-ER
Rock2
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control miR-181a-5p mimic
E
RBE-ER
Relative expression of miR-181-5p
Control
miR-181a-5p mimic
F
RBE-ER
Relative expression of ROCK2 mRNA
Control
miR-181a-5p mimic
G
9810-ER
Relative expression of miR-181-5p
Control
miR-181a-5p mimic
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9810-ER
Relative expression of ROCK2 mRNA
Control
miR-181a-5p mimic
I
RBE-ER
Vector
ROCK2
miR-181a-5p mimics
ROCK2+miR-181a-5p mimics
Relative luciferase activity
NS
ROCK2 WT
ROCK2 Mut
J
9810-ER
Vector
ROCK2
miR-181a-5p mimics
ROCK2+miR-181a-5p mimics
Relative luciferase activity
NS
ROCK2 WT
ROCK2 Mut
K
RBE-ER
Relative expression of CCAT1
shNC
shCCAT1
RBE-ER
Relative expression of ROCK2 mRNA
shNC
shCCAT1
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9810-ER
Relative expression of CCAT1 mRNA
shNC
shCCAT1
9810-ER
Relative expression of ROCK2 mRNA
shNC
shCCAT1
M
RBE-ER
shNC
shCCAT1
miR-181a-5p inhibitor
shCCAT1+miR-181-5p inhibitor
Relative luciferase activity
NS
CCAT1 WT
CCAT1 Mut
N
9810-ER
shNC
shCCAT1
miR-181a-5p inhibitor
shCCAT1+miR-181-5p inhibitor
Relative luciferase activity
NS
CCAT1 WT
CCAT1 Mut
O
RBE-ER
Rock2
160kD
Tublin
55kD
shNC
shCCAT1
miR-181a-5p inhibitor
shCCAT1+miR181a-5p inhibitor
P
9810-ER
Rock2
160kD
Tublin
55kD
shNC
shCCAT1
miR-181a-5p inhibitor
shCCAT1+miR181a-5p inhibitor
Q
NC
shCCAT1
miR-181a-5p inhibitors
shCCAT1+ miR-181a-5p inhibitors
Merge
Cy3
DAPI
RBE-ER
R
NC
shCCAT1
miR-181a-5p inhibitors
shCCAT1+ miR-181a-5p inhibitors
Merge
Cy3
DAPI
9810-ER

Figure 6. CCAT1 functions as a competing endogenous RNA for miR-181a-5p to facilitate ROCK2 expression in ER CCA cells. (A and B) Schematic representation of binding sequences of CCAT1 (A) and ROCK2 (B) promoter and miR-181a-5p predicted by microRNA bioinformatic databases (Targetscan and Star-base v2.0). (C and D) Western blot analyses were performed to detect ROCK2 protein expression levels in RBE-ER (C) and 9810-ER (D) cells transfected with miR-181a-5p mimic. (E and F) qRT-PCR analysis of miR-181a-5p (E) and ROCK2 (F) expression in RBE-ER cells with miR-181a-5p mimic. (G and H) qRT-PCR analysis of miR-181a-5p (G) and ROCK2 (H) expression in 9810-ER cells with miR-181a-5p mimic. (I and J) A ROCK2 promoter or the same promoter with mutation sequences reporter luciferase assay was performed using RBE-ER (I) and 9810-ER (J) cells transfected with the indicated plasmid or/and miR-181a-5p mimic. (K and L) qRT-PCR analysis of CCAT1 and ROCK2 expression in RBE-ER (K) and 9810-ER (L) cells transfected with shCCAT1. (M and N) A CCAT1 promoter or the same promoter with mutation sequences reporter luciferase assay was performed using RBE-ER (M) and 9810-ER (N) cells transfected with the indicated plasmid or/and miR-181a-5p inhibitor. (O and P) Western blot analyses were performed to detect ROCK2 protein expression levels in RBE-ER (O) and 9810-ER (P) cells transfected with the indicated plasmid or/and miR-181a-5p inhibitor. (Q and R) Cell apoptosis was measured using TUNEL assays in RBE-ER (Q) and 9810-ER (R) cells transfected with the indicated plasmid or/and miR-181a-5p inhibitor.

tion between CCAT1 and erlotinib resistance in CCA has not been investigated, making this the first study to do so. We showed that CCAT1 expression was considerably higher in ER CCA cells, and it was significantly correlated with erlotinib resistance. *In vitro* and *in vivo* experiments further demonstrated that downregulating CCAT1 slowed the development of CCA cells and made them more responsive to erlotinib treatment, suggesting that CCAT1 could be used as a biomarker of erlotinib sensitivity in CCA.

EMT serves a critical role in mediating erlotinib resistance. For instance, in non-small cell lung carcinoma, guanylate-binding protein 1 stimulated erlotinib resistance through phosphoglycerate kinase 1-activated EMT signaling [32]. Cufí S et al. showed that silibinin reversed the elevated miR-21 profile, thereby suppressing EMT-driven erlotinib resistance [33]. Furthermore, the lncRNA CCAT1 has been shown to induce EMT in various tumor types. For instance, CCAT1 was shown to sponge miR-218-5p to facilitate EMT in retinoblastoma via metal response element-binding transcription factor 2 [34]. Mu Y et al. demonstrated that CCAT1 upregulated TGF-β receptor 1 in ovarian cancer cells to facilitate EMT via TGF-β1 [35]. Therefore, we speculated whether CCAT1 regulated erlotinib resistance in CCA by inducing EMT. We found that in ER CCA cells, EMT was reversed when CCAT1 was silenced; however, this reversal was not observed in the presence of an EMT activator. Thus, these findings indicate that CCAT1 modulated EMT to influence the erlotinib sensitivity of CCA cells.

ROCK2 is a well-known regulator of EMT. According to Cao et al., osteopontin promoted EMT and the cancer stem cell-like characteristics of pancreatic cancer cells by activating the integrin αvβ3-Akt/extracellular signal-regulated kinase-forkhead box protein M1 cascade in a paracrine manner [36]. Similarly, Huang et al. discovered that forkhead box protein M1 directly stimulated the transcription of caveolin-1 to induce EMT and metastasis in pancreatic cancer [37]. The non-coding RNA nc886 was shown to enhance EMT via the ROCK2 phosphorylation-mediated nuclear translocation of β-catenin, which in turn facilitated drug resistance in renal cancer cells [38]. In this study, we discovered that silencing ROCK2 enhanced the drug sensitivity of ER CCA cells, but this was inhibited when an EMT activator was added, indicating that ROCK2 regulated EMT to induce erlotinib resistance in CCA cells. However, the upstream mechanism by which ROCK2 modulates erlotinib resistance in CCA cells remains unclear. ROCK2 was downregulated in CCAT1-knockdown ER CCA cells. Further, ROCK2 could reverse the changes effected by CCAT1 knockdown, highlighting that it was a key protein in CCAT1's regulation of EMT leading to erlotinib resistance in CCA. Next, we investigated the mechanism whereby CCAT1 regulated ROCK2. The expression of ROCK2 decreased when miR-181a-5p was overexpressed. The luciferase activity of the WT 3' UTR of ROCK2 was attenuated by miR-181a-5p, but not that of the MUT 3' UTR of ROCK2. miR-181a-5p was downregulated when CCAT1 was overexpressed. These observations demonstrated that miR-181a-5p could bind to CCAT1, and that miR-181a-5p amplified the inhibitory effects of CCAT1 overexpression on ROCK2 while reversing its stimulatory effects on CCA cell proliferation. Furthermore, CCAT1 deficient in the miR-181a-5p binding domain failed to promote pro-

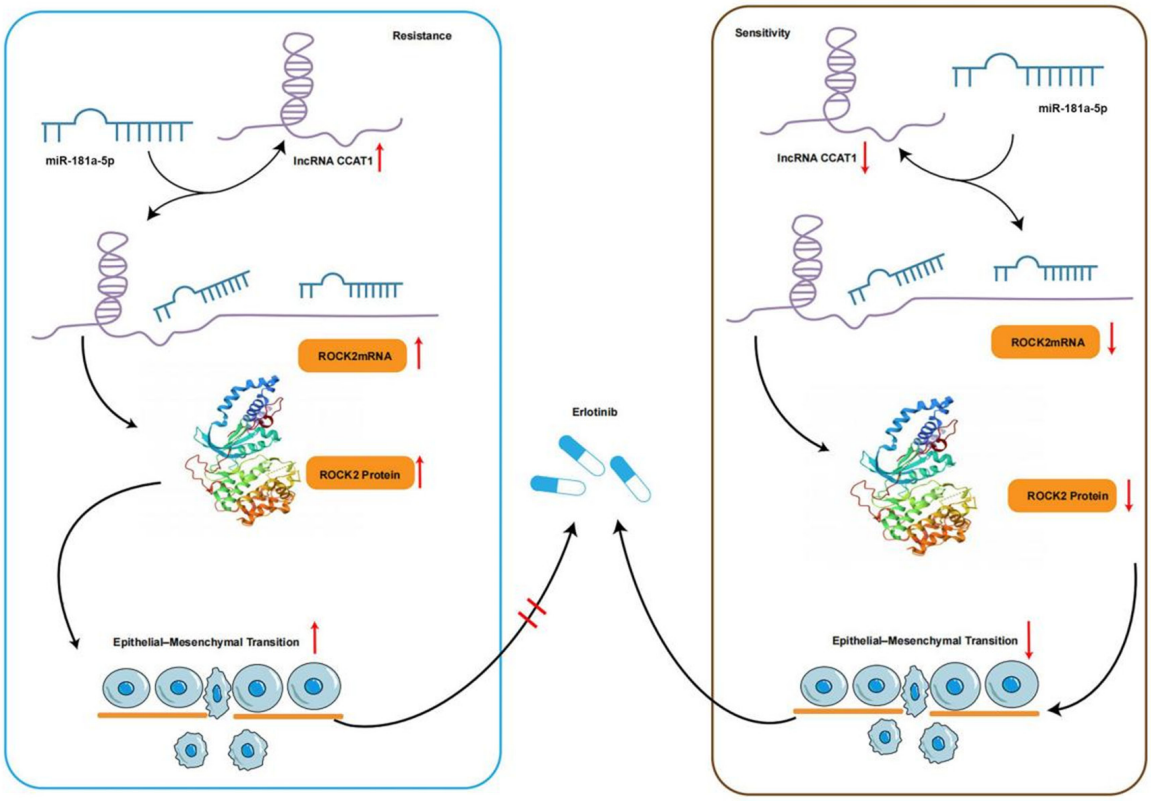

Figure 7. Proposed model by which lncRNA CCAT1 promotes erlotinib resistance in cholangiocarcinoma by inducing epithelial-mesenchymal transition via the miR-181a-5p/ROCK2 axis. lncRNA CCAT1 expression was considerably higher in ER CCA cells, and it was significantly correlated with erlotinib resistance. The functional suppression of CCAT1 holds promise in enhancing the sensitivity to erlotinib by reversing EMT through the miR-181a-5p/ROCK2 signaling pathway.

liferation. Overall, these data demonstrated that the lncRNA CCAT1 regulated ROCK2 to promote erlotinib resistance in CCA by inducing EMT via miR-181a-5p.

In this study, we first reported that the lncRNA CCAT1 was highly expressed in CCA, and knocking it down reversed erlotinib resistance in CCA cells. Through *in vitro* experiments, we showed that upregulating ROCK2 restored the changes in the expression of EMT-associated proteins induced by CCAT1 knockdown, and downregulating ROCK2 reversed CCAT1 overexpression. These findings suggest that the lncRNA CCAT1 causes erlotinib resistance in CCA cells, and ROCK2 plays a major role in this process. Lastly, we investigated the mechanism through which CCAT1 modulated ROCK2. We discovered that miR-181a-5p could bind to CCAT1 to regulate ROCK2 expression (**Figure 7**). In conclusion, we proved that the lncRNA CCAT1 enhanced erlotinib resistance in CCA by inducing EMT via the miR-181a-5p/ROCK2 axis.

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Disclosure of conflict of interest

None.

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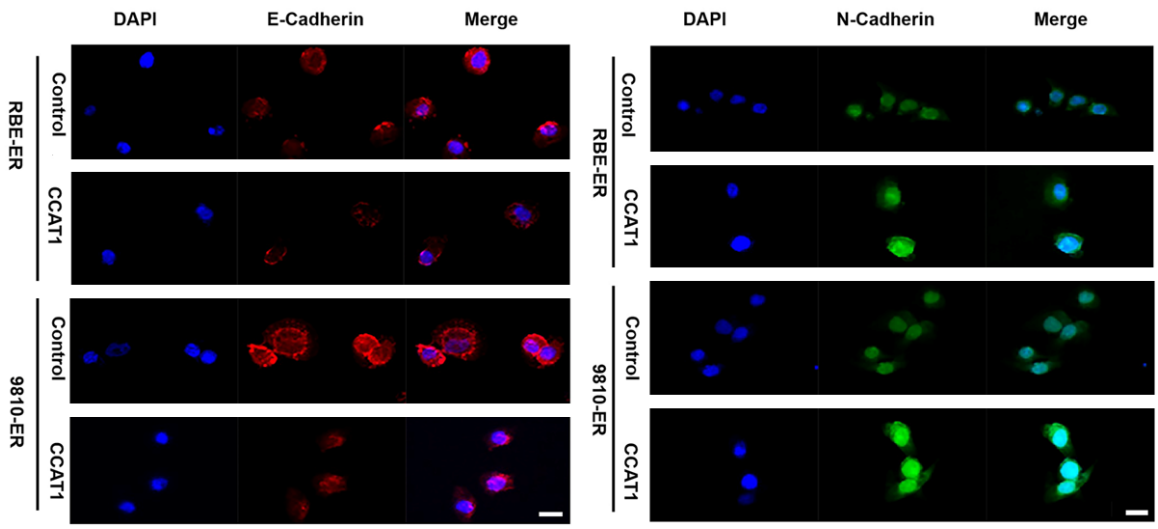
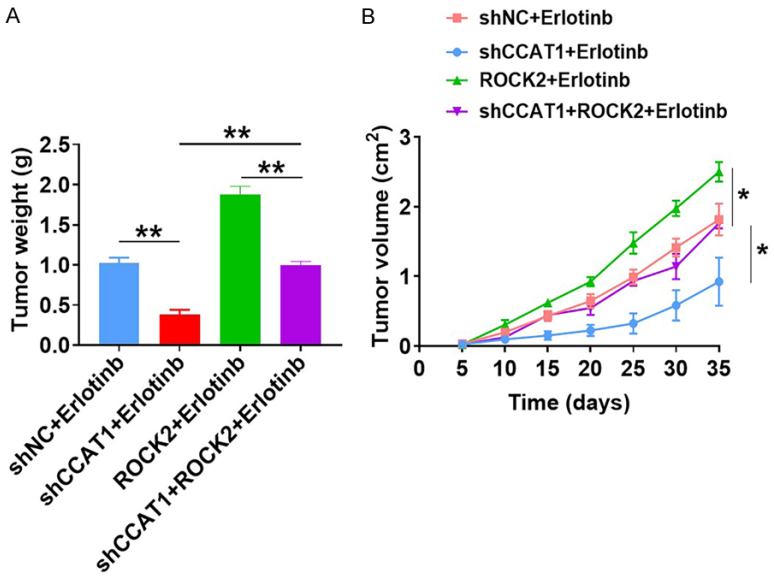


Supplementary Figure 1. CCAT1 regulates EMT to promote erlotinib resistance in CCA cells. Immunofluorescence analysis of E-cadherin and N-cadherin expression in CCAT1-CCA cells.



Supplementary Figure 2. CCAT1 regulates EMT to promote erlotinib resistance in CCA cells. A and B. The quantification of tumour volume or tumour weight assay in the different groups. *P < 0.05.