

Original Article

LncRNA CCAT1 regulates ADAM8 via binding miR-6855-5p to promote progression in colorectal cancer cell RKO

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Received May 24, 2020; Accepted September 29, 2020; Epub December 15, 2020; Published December 30, 2020

Abstract: The function and clinical relevance of colon cancer-associated transcript-1 (CCAT1) lncRNA in colorectal cancer (CRC) oncogenesis are still unclear. In our previous study, we found that CCAT1 was highly expressed in CRC tissues and colorectal cancer cell line RKO cells, and promoted the proliferation and invasion of RKO. Here, we knocked-down the expression of CCAT1 in RKO cells using CCAT1-specific siRNAs, and applied total transcriptome and mRNA sequencing techniques, analyzed the genes differentially expressed following CCAT1 knock-down and verified the possible signaling pathway of CCAT1 in CRC by western blotting and luciferase reporter assay. The total transcriptome and mRNA sequencing results showed that CCAT1 plays a regulatory role in RKO cells by modulating the expression of a disintegrin and metalloprotease 8 (ADAM8) via binding to miR-6855-5p. Luciferase reporter assay confirmed the presence of a CCAT1/miR-6855-5p/ADAM8 signaling pathway in RKO cells. We further verified the activity of this signaling pathway *in vitro* and *in vivo* using western blotting and RT-qPCR analyses. Our results demonstrated that CCAT1 functions as an oncogenic lncRNA in RKO cells and could thus serve as a potential diagnostic and therapeutic target for CRC patients.

Keywords: CCAT1, colorectal cancer, miR-6855-5p, ADAM8, RKO

Introduction

The incidence rate and mortality of colorectal cancer (CRC) are growing significantly worldwide, with CRC currently being a leading cause of disease-related death. More than 376,000 people are newly diagnosed with CRC each year, with the disease causing an annual death rate of approximately 190,000 in China [1]. Surgical removal is considered to be the only possible cure for colorectal cancer. However, due to the lack of universal screening techniques and awareness regarding colorectal cancer, most new cases are diagnosed at the advanced stage [2]. Therefore, preoperative and postoperative adjuvant chemotherapy have become essential to improve the disease-free survival and increase the five-year survival rate of colorectal cancer patients. Thus, it is necessary and crucial to find effective diagnos-

tic markers and to identify valid therapeutic targets for CRC.

Numerous studies have shown that long non coding RNAs (lncRNAs) that are abnormally expressed in tumors play a critical role in tumor initiation and progression, which makes lncRNAs valuable diagnostic biomarkers and therapeutic targets for cancer [3-5]. For example, Chen et al. indicated that lncRNA SNHG-16 promotes hepatocellular carcinoma growth, migration, and invasion by regulating miR-186 expression [6]. However, the clinical significance of many unique lncRNAs and the underlying molecular mechanisms of their functions are unclear. Multiple studies have provided increasing evidence that lncRNA CCAT1 is highly expressed in many tumors and suggesting a role in these cancer development [7-9]. For example, Hu et al. found that the lncRNA

CCAT1/mir-130a-3p axis mediates cisplatin resistance in non-small-cell lung cancer cell lines by targeting SOX4 [10]. We previously showed that CCAT1 promoted the proliferation and invasion of colorectal cancer cell line RKO cells. Here, we further explored the potential mechanism of CCTA1 in RKO cells.

Materials and methods

Cell culture

For this study, we used the human colorectal cancer cell line RKO. The cell line was provided by the Chinese Academy of Science (Shanghai, China), and cells were cultured in 90% DMEM (Gibco, ThermoFisher Scientific, China) containing 10% FBS (Gibco, Thermo Fisher Scientific, China) with 1% penicillin and streptomycin (Gibco, Life Technologies, China). RKO cells were propagated at 37°C in a humidified atmosphere containing 5% CO₂.

RNA preparation and RT-qPCR

Total RNA was extracted from both tissues and cells using the RNA rapid extraction kit (Aidlab Biotechnologies, Co, RN28, China), according to the manufacturer's instructions. RNA was reverse-transcribed into cDNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, RR047). RT-qPCR was performed using TB Green® Premix Ex Taq™ (Tli RNaseH Plus) (Takara, RR420). The final gene expression levels were normalized to the expression level of the housekeeping gene GAPDH in each sample. Primer sequences used in this study are shown in [Supplementary Table 1](#). All primers were synthesized by Beijing tsingke biological technology, China. All RTqPCR reactions were performed on the ABI7500 system (Applied Biosystems, Carlsbad, CA, USA) with three repeats.

Cell transfection

CCAT1-specific siRNAs were synthesized by GenePharma, Shanghai, China. The siRNA sequences used in this experiment are shown in [Supplementary Table 1](#). The Lipofectamine 2000 kit (Invitrogen, USA) was used for transfection according to the manufacturer's instructions. Twenty-four hours after transfection, the total RNA of the cells was collected, and

the transfection efficiency was evaluated using RT-qPCR.

Cell cycle assay

The cells were collected and washed twice with cold PBS. After addition of 5 ml pre-cooled 75% ethanol, the samples were stored at 4°C overnight. After centrifugation at 2000 rpm, the cells were washed twice with PBS and resuspended in 0.5 ml PI/RNase Staining Buffer Solution (BD biosciences, China). Following 15 minutes incubation in the dark at 25°C, the samples were subjected to the cell cycle analysis using a flow cytometer (BD biosciences, China).

Annexin V-PI double staining apoptosis assay

To exclude the effect of RKO apoptosis on the results of the CCK-8 assay, Annexin V-PI double staining was performed to detect the apoptosis before and after transfection of RKO cells. For this experiment, FITC Annexin V Apoptosis Detection Kit I (BD biosciences, China) was used. Twenty-four hours after transfection, RKO cells + si-RNA and NC groups were collected as described above. After washing the cells twice with cold PBS, the cell density was adjusted to 1×10^6 cells/ml using $1 \times$ Binding Buffer. Then, 100 µl of the suspension (1×10^5 cells) was transferred to a 5 ml flow cytometry tube, where 5 µl of FITC Annexin V and 5 µl PI were subsequently added. Each tube was supplemented with 400 µl $1 \times$ Binding Buffer and measured using a flow cytometer (BD Biosciences, China) within 1 h.

Western blot analysis

Lysis buffer (FD Bioscience, China) was used to extract proteins for the western blot analysis. Appropriate amounts of protein lysates were electrophoresed using 10% SDS-PAGE and transferred to a Immobilon-PSQ membrane. After blocking with bovine serum albumin (BSA) for 1 h, the membrane was incubated at 4°C for 1 h with ADAM8 (RABMAB, China) or GAPDH (RABMAB, China) primary antibodies diluted to 1:1000 with 5% buffer solution. After two washes with PBS, the membrane was cultured with RABBITt-HRP (FD Bioscience, China) secondary antibody at 25°C for 1 h. Dura ECI KIT (FD Bioscience, China) and Image J were used to detect protein expression.

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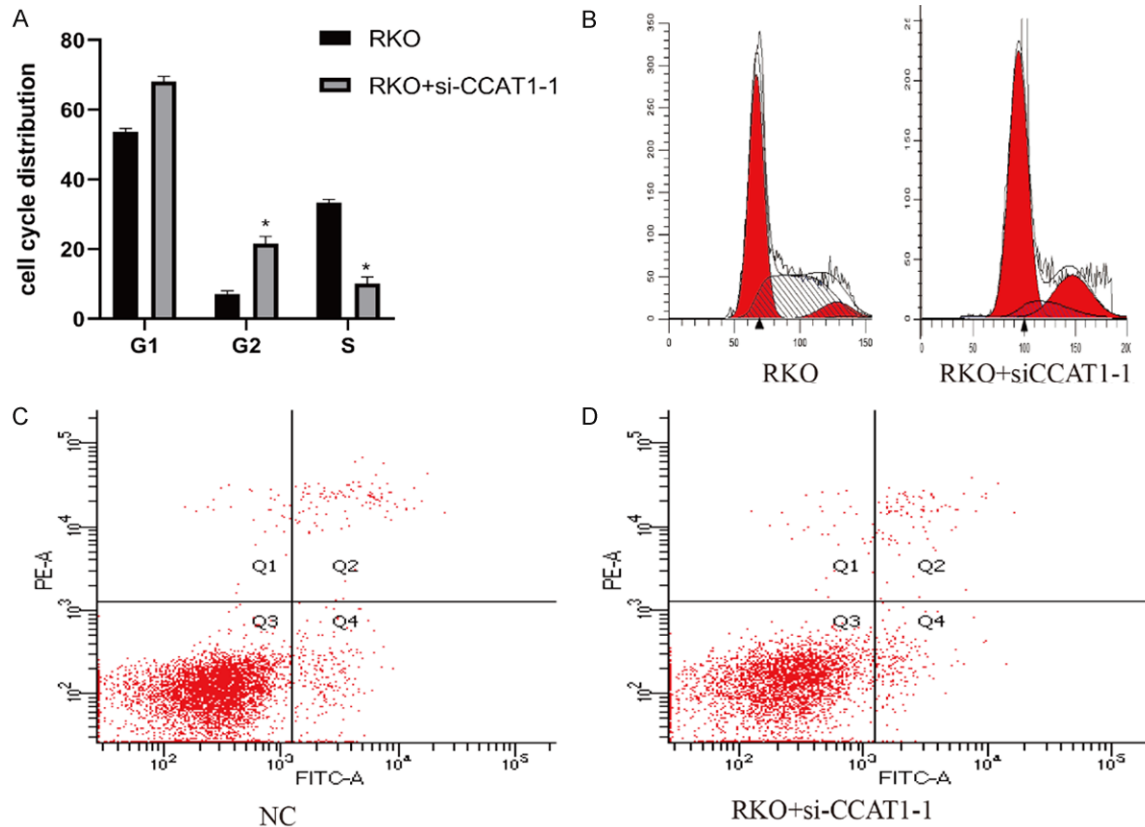


Figure 1. CCAT1 promoted RKO cell proliferation. A. Cell cycle distribution in RKO cells + si-RNA and NC groups. B. Cell cycle distribution of RKO cells and RKO cells + si-CCAT1-1. C. Results of apoptosis detection in the NC-group. D. Results of apoptosis detection in RKO cells + si-CCAT1-1. *P < 0.05, **P < 0.01. NC, untreated RKO cells.

Luciferase reporter assay

The sequences of miR-6855-5p corresponding to ADAM8 wild-type and mutant cDNA were subcloned into the pmirGlo vector. Two sequences of the miR-6855-5p inhibitor were cloned one after another into the pmirGlo vector as the positive control for the experiment (PC). The experiments were performed using RKO cells and groups were made as follows: A. mimic NC + ADAM8 WT (negative control miRNA with wild-type ADAM8 cDNA in the expression vector); B. miR-6855-5p mimic + ADAM8 WT (miR-6855-5p mimic with wild-type ADAM8 cDNA in the expression vector); C. mimic NC + ADAM8 MUT (negative control miRNA with mutant-type ADAM8 cDNA in the expression vector); D. miR-6855-5p mimic + ADAM8 MUT (miR-6855-5p mimic with mutant-type ADAM8 cDNA in the expression vector); E. mimic NC + pmirGlo PC (negative control miRNA with pmirGlo PC); F. miR-6855-5p mimic + pmirGlo PC (miR-6855-5p mimic with pmirGlo PC). Twenty-

four hours after transfection, luciferase activity was detected using the Tecan M1000 multifunctional enzyme-labeled device.

Statistical analysis

GraphPad Prism 8.0.1 (GraphPad Software, USA) was used for data analysis. All the experiments were carried out at least three times. Data were presented as mean \pm SD and P < 0.05 was considered a significant difference. For evaluation of RT-qPCR results, the relative expression levels of CCAT1 were calculated via $2^{-\Delta\Delta Ct}$ method.

Results

CCAT1 promoted RKO cell proliferation

We found that after knock-down of CCAT1 expression, RKO cells were significantly arrested in the G2 phase (P < 0.05) (Figure 1A, 1B). We further conducted an apoptosis assay to exclude the possible effects of RKO apoptosis.

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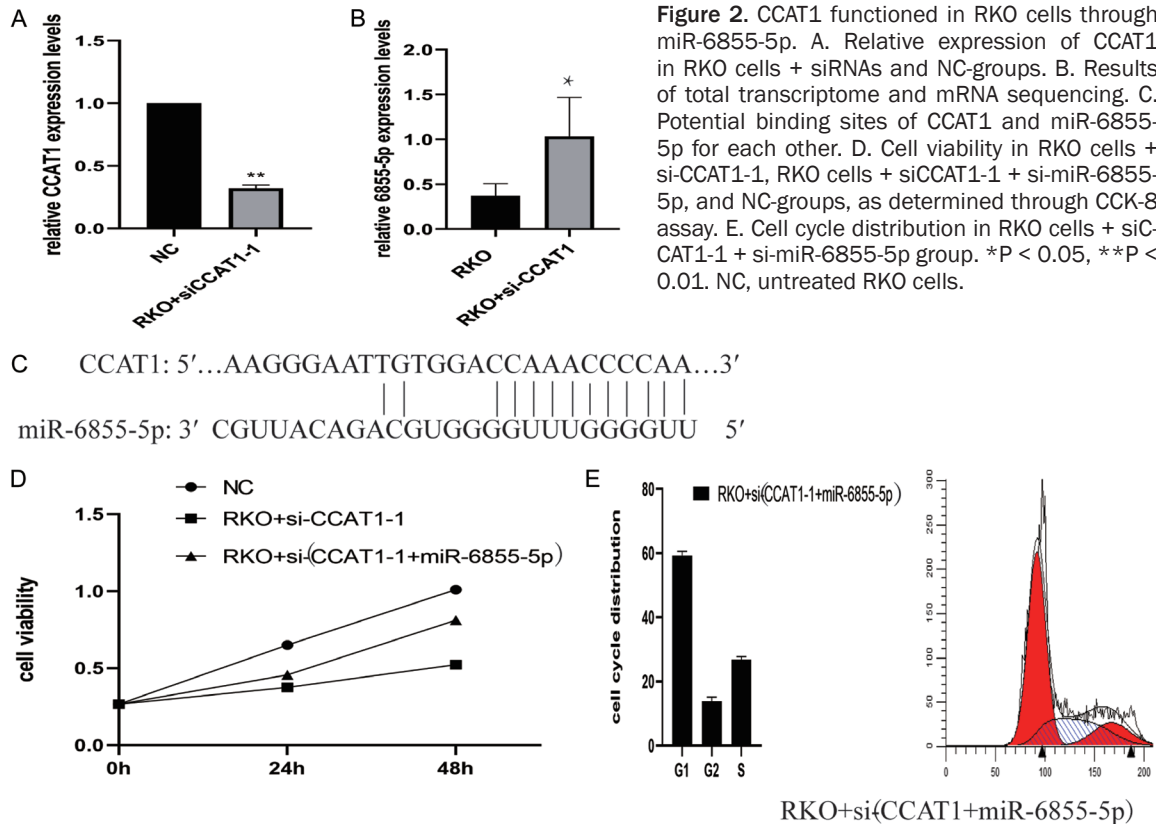


Figure 2. CCAT1 functioned in RKO cells through miR-6855-5p. A. Relative expression of CCAT1 in RKO cells + siRNAs and NC-groups. B. Results of total transcriptome and mRNA sequencing. C. Potential binding sites of CCAT1 and miR-6855-5p for each other. D. Cell viability in RKO cells + si-CCAT1-1, RKO cells + siCCAT1-1 + si-miR-6855-5p, and NC-groups, as determined through CCK-8 assay. E. Cell cycle distribution in RKO cells + si-CCAT1-1 + si-miR-6855-5p group. * $P < 0.05$, ** $P < 0.01$. NC, untreated RKO cells.

There was no significant difference in the apoptosis of RKO cells before and after CCAT1-specific siRNA transfection ($P > 0.05$) (Figure 1C, 1D). Thus, CCAT1 may play a role in promoting proliferation of RKO cells.

CCAT1 activity in RKO cells was mediated by miR-6855-5p

Currently, many studies suggest that lncRNAs play a role as competing endogenous RNAs (ceRNAs) for miRNAs [11]. To identify the signaling pathway of CCAT1 in colorectal cancer, we conducted total transcriptome and mRNA gene sequencing. As shown in Figure 2A, we efficiently silenced CCAT1 expression in the experimental group of the RKO cell line ($P < 0.01$); this was confirmed by PCR analysis. Using second-generation gene sequencing, we analyzed the genes differentially expressed in RKO cells after transfection with CCAT1-specific siRNA. As shown in Figure 2B, we found that the expression of miR-6855-5p was significantly increased after CCAT1 silencing ($P < 0.01$). Furthermore, CCAT1 and miR-6855-5p base arrangements were analyzed using the BioEdit software. As shown in Figure 2C, CCAT1 and miR-6855-5p have potential binding sites

for each other. Therefore, we then conducted a rescue assay. After downregulation of CCAT1 expression, we observe reduced expression of miR-6855-5p. As shown in Figure 2D, 2E, partially improved proliferation capacity of RKO cells and the population of RKO cells stagnating in the G2 phase was detected. These findings strongly suggested that CCAT1 is involved in CRC pathogenesis by binding to miR-6855-5p.

ADAM8 was identified as the target of miR-6855-5p

As shown in Figure 3A, total transcriptome and mRNA sequencing revealed many mRNAs with significantly changed levels in RKO cells. One of the prominent candidate differentially expressed genes was ADAM8 ($P < 0.05$). As shown in Figure 3B, we analyzed the base arrangements of ADAM8 and miR-6855-5p using the BioEdit software and the online bioinformatics software TargetScan and found each to have a potential binding site for the other. Therefore, we speculated that ADAM8 might be the target of miR-6855-5p. To further confirm our hypothesis, we conducted a luciferase reporter assay. Compared with the mimic

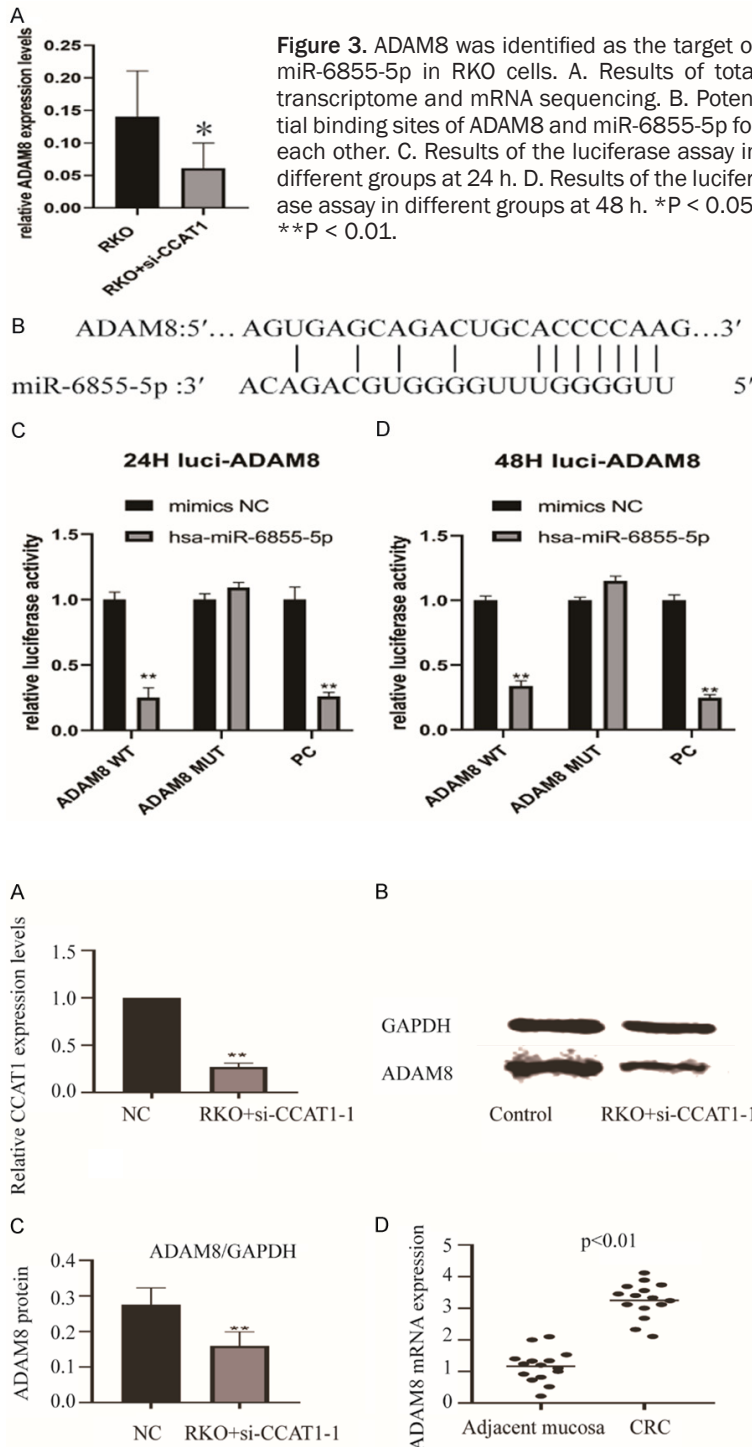


Figure 4. ADAM8 was highly expressed in CRC in vitro and in vivo. A. Relative expression of CCAT1 in RKO cells + si-CCAT1-1 and NC groups. B. The expression of ADAM8 in the NC groups and RKO cells + si-CCAT1-1 groups. C. Relative expression of ADAM8 protein in NC and RKO cells + si-CCAT1-1 groups using qRT-PCR-based detection of ADAM8/GAPDH. D. Relative expression of ADAM8 mRNA in CRC and adjacent mucosa. **P < 0.01.

NC+ADAM8 WT group, the luciferase activities of miR-6855-5p+ADAM8 WT and PC groups

were significantly reduced (P < 0.01) (**Figure 3C**). This proved that miR-6855-5p targets ADAM8 and affects its functions.

ADAM8 was highly expressed in CRC in vitro and in vivo

In order to confirm that ADAM8 plays a role in the signaling pathway involving CCAT1, we silenced the expression of CCAT1 in RKO cells (**Figure 4A**) and conducted a western blot analysis. As shown in **Figure 4B, 4C**, knock-down of CCAT1 caused a decrease in ADAM8 expression compared with that in the control group (P < 0.01). We also performed PCR with the cancer and its adjacent tissues from 15 patients with colorectal cancer who had not received preoperative chemoradiotherapy. As shown in **Figure 4D**, the ADAM8 expression level in cancer tissues was significantly higher than that in adjacent tissues (P < 0.01). These data indicate that CCAT1 finally acted on ADAM8 and played a role in the initiation and progression of CRC.

CCAT1 regulated ADAM8 by binding miR-6855-5p and was related to poor prognosis

To explore the mechanism of CCAT1 activity in CRC, we performed full transcriptome and mRNA gene sequencing, double luciferase reporter gene assay, RT-qPCR, and western blot analysis. We found that CCAT1 plays a regulatory role through the signaling pathway involving the CCAT1/miR-6855-5p/ADAM8 axis. Furthermore, using the FunRich software and OncoLnc (<http://www.oncolnc.org/>), we found that somatic mutation in ADAM8 is

significantly associated with colorectal cancer (P < 0.05) (**Figure 5A**) and that high ADAM8

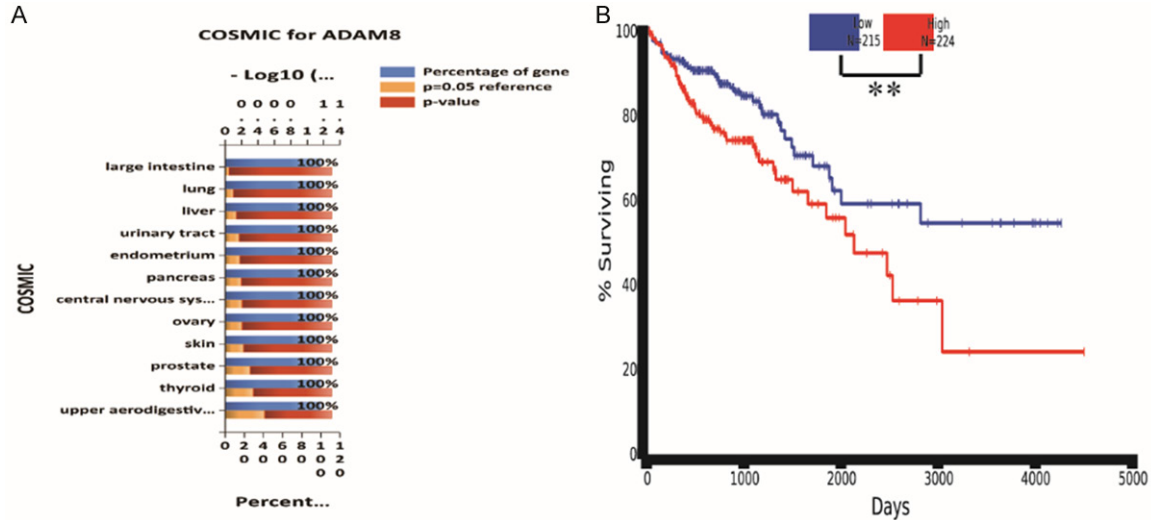


Figure 5. CCAT1 regulated ADAM8 via binding miR-6855-5p and was related to poor prognosis. A. Output of The Catalogue of Somatic Mutations in Cancer (COSMIC) for ADAM8 using the FunRich Software. B. Survival rate of cells with low and high expression of ADAM8 analyzed using OncoLnc (<http://www.oncolnc.org/>). **P < 0.01.

expression is related to shorter overall survival (P < 0.01) (Figure 5B).

Discussion

Presently, lncRNAs are known to regulate the expression of target genes by sponging miRNAs and are thus defined as competing endogenous RNAs (ceRNAs) [12, 13]. These ceRNAs are involved in the biological behavior of tumors and play a key role in tumor progression [14]. Jia Y et al. found that lncRNA TTN-AS1 promotes the migration, invasion, and epithelial-mesenchymal transition of lung adenocarcinoma cells via the miRNA-142-5p sponging-mediated regulation of CDK5 [15]. Moreover, Zhao B et al. demonstrated that miRNA-124 inhibits the proliferation, migration, and invasion of hepatocellular carcinoma cells by downregulating lncRNA-UCA1 [16]. Our study confirmed that CCAT1 might regulate the expression of the target gene ADAM8 in RKO cells as a ceRNA of mir-6855-5p.

ADAM8 is a member of the human A disintegrin and metalloprotease family, which contains disintegrin and metalloprotease domains [17]. A large number of studies have shown that ADAM8 is highly expressed in many tumors and is closely related to tumor metastasis and poor prognosis [18-20]. For example, Liu et al. found that ADAM8 promotes chondrosarcoma cell migration and invasion via activation of the

NF-kappaB/MMP-13 signaling axis [21]. According to the present study, ADAM8 is mainly involved in the activation of adhesion molecules [22], promotion of the degradation of ECM, interaction with integrin [23], and stimulation of angiogenesis [24] to promote tumor metastasis.

Overall, in our study, we found that CCAT1 promotes the proliferation, migration, and invasion of CRC cells. Through total transcriptome and mRNA sequencing, we identified that CCAT1 plays a critical role in CRC via sponging miR-6855-5p to regulate ADAM8. Furthermore, we found that the high expression of ADAM8 was associated with poor prognosis in CRC. These results may provide new insights for the development of novel diagnostic markers and treatment strategies for CRC.

Acknowledgements

The Project was supported by Zhejiang Provincial Natural Science Foundation of China. [Y18H160291].

Disclosure of conflict of interest

None.

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Supplementary Table 1. Prime sequences and si-RNA sequences used in this study

	Forward (5'-3')	Reverse (5'-3')
GAPDH	GGTATCGTGGAAGGACTCATGAC	ATGCCAGTGAGCTTCCCGTTCAG
CCAT1	TCACTGACAACATCGACTTTGAAG	GGAGAAAACGCTTAGCCATACAG
ADAM8	CTACACAGAGACCTATACGGC	CACCTGGAAGAAACCCCTGA
Si-CCAT1-1	UUCGUUUACUUAACAGGGCTT	
Si-CCAT1-2	AGUCAAAUCAGAUCCGUGCTT	
Si-CCAT1-3	UCAAGCAUAAAUGGGACAGTT	
Si-miR-6855-5p	GCAAUGUCUGCACCCCAACCCCAA	