

Upregulation of long noncoding RNA CCAT1-L promotes epithelial–mesenchymal transition in gastric adenocarcinoma

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Objective: In this study, we aimed to investigate the role of a long-chain noncoding RNA, colorectal cancer-associated transcript 1-long (CCAT1-L) in gastric adenocarcinoma.

Patients and methods: Expressions of CCAT1-L and c-MYC mRNA and MYC protein in gastric adenocarcinoma tissue and adjacent normal tissues of 60 patients were analyzed using quantitative real-time polymerase chain reaction and Western blot, respectively. The CCAT1-L levels in the normal gastric epithelial cell line, GES1, and human gastric adenocarcinoma cell lines, MGC803, MKN-28, SGC7901, and BGC823 were analyzed by quantitative real-time polymerase chain reaction. CCAT1-L knockdown in MGC803 and MKN28 cells was performed using RNA interference, followed by evaluating cell proliferation, invasion, and migration with soft agar colony formation assay, scratch wound assay, and transwell assay. Twenty BALB/C-nu-nu nude mice were inoculated with gastric tumor xenografts and treated with CCAT1-L small-interfering RNA (siRNA), followed by monitoring survival and tumor growth. Western blot was also used to analyze the expression of epithelial–mesenchymal transition-related proteins, including MYC, RAS, T-ERK, P-ERK, E-cadherin, and vimentin, in gastric adenocarcinoma MKN-28 cells.

Results: The expression of CCAT1-L and MYC in tumor tissue was significantly higher than that in adjacent normal tissues ($P < 0.001$). There was a positive correlation between the expression level of CCAT1-L mRNA and c-MYC mRNA ($r = 0.863$, $P < 0.001$). CCAT1-L expression was also significantly higher in gastric adenocarcinoma cell lines than that in normal cell lines ($P < 0.01$). Knockdown of CCAT1-L in MGC803 and MKN-28 cells markedly reduced the cell proliferation, migration, and invasion ($P < 0.001$). CCAT1-L knockdown also evidently inhibited tumor growth and improved survival in nude mice ($P < 0.001$). Expressions of MYC, RAS, and vimentin, and the phosphorylation of ERK protein were dramatically decreased, while the expression of E-cadherin protein was increased by CCAT1-L knockdown in MKN-28 cell.

Conclusion: CCAT1-L is a pro-oncogenic marker in gastric adenocarcinoma. CCAT1-L knockdown inhibits epithelial–mesenchymal transition of gastric adenocarcinoma cells and thus suppresses the gastric adenocarcinoma metastasis.

Keywords: lncRNA CCAT1-L, MYC, gastric adenocarcinoma, EMT

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Introduction

Gastric cancer is one of the most common cancers across the globe and the second leading cause of cancer-related death. The incidence rate of gastric cancer is particularly high in East Asia and South America.¹ Apart from common risk factors, such as *Helicobacter pylori* infection, several genetic alterations have been linked with the development of gastric adenocarcinoma.² A major class of genetic

alterations are pro-oncogenic processes, for example epithelial–mesenchymal transition (EMT), which promote the loss of cell-to-cell adhesion and acquisition of cell-to-matrix interaction.³ Studies have focused on the search of specific molecules related to EMT for the detection and therapy of gastric cancer.³

There is growing evidence that long-chain noncoding RNAs (lncRNAs) play a crucial role in a large variety of biological processes.⁴ lncRNAs are a class of regulatory noncoding RNAs with the length of over 200 nucleotides. Aberrant expression of lncRNAs is a notorious contributor to cancers.^{5–7} Colorectal cancer-associated transcript 1 (CCAT1) is a highly expressed lncRNA in colon cancer and other cancers.^{8,9} It is located on the 8q24 region of the human chromatin. Recently, a high CCAT1 expression was also documented in human gastric adenocarcinomas, indicating that CCAT1 may be a molecular marker of gastric adenocarcinoma.² Recently, Xiang et al discovered a new type of CCAT1, which was named CCAT1-L (CCAT1-Long) because of its greater length compared with the previously reported CCAT1.¹⁰ CCAT1-L is located at 515 kb upstream of the MYC gene and regulates the expression of MYC gene by mediating the formation of a loop between the MYC promoter and enhancer, thereby affecting the development of colon cancer.¹⁰ Abnormal expression of CCAT1-L not only causes colon cancer, but also leads to gastric carcinogenesis.² Therefore, CCAT1-L may be a molecular marker in gastric adenocarcinoma, and therapeutic strategies may be developed by targeting CCAT1-L.

Herein, we aimed to study the role of CCAT1-L in the development and progression of gastric adenocarcinoma. We used siRNA¹¹ to knockdown CCAT1-L to study its role in cancer proliferation, migration, and invasion. The interaction of CCAT1-L with EMT proteins is also investigated. The results of this study could provide a new tool for the diagnosis and treatment of gastric cancer.

Patients and methods

Patients

Sixty cases of gastric adenocarcinoma tissue and adjacent normal tissues were enrolled in The Affiliated Yantai Yuhuangding Hospital of Qingdao University. All patients were confirmed with pathological examination. All tissues were stored in liquid nitrogen tank after surgical resection. The sample collection was approved by the patients, and all patients gave written informed consent. The study was approved by the ethics committee of The Affiliated Yantai Yuhuangding Hospital of Qingdao University.

Quantitative real-time PCR

TRIzol kit (Takara, Dalian, China) was used to extract the total RNA from tumor tissue of gastric adenocarcinoma and adjacent healthy tissue after homogenization. RNA was then transcribed into cDNA using the reverse transcription kit (Applied Biosystems, Waltham, MA, USA). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using Mastercycler[®] nexus X2 (Eppendorf, Hamburg, Germany). The data were analyzed using the $2^{-\Delta\Delta C_t}$ method,¹² and the relative expression levels were calculated using GAPDH mRNA as an internal control. Primer sequences used in this study are as follows: CCAT1-L, forward: 5'-CCAC GTGCACATATTTGAATTG-3' and reverse: 5'-TGCATT CCCTGCTTAATACTCA-3'. c-MYC, forward: 5'-CACAG CAAACCTCCTCACAG-3' and reverse: 5'-GGATAG TCCTTCCGAGTGGA-3'. GAPDH, forward: 5'-AGCCCA TCACCATCTTCCAG-3' and reverse: 5'-CCTGCTT CACCACCTTCTTG-3'.

Cell culture

The normal human gastric epithelial cell line GES1, human gastric adenocarcinoma cell lines MGC803, MKN-28, SGC7901, and BGC823 were purchased from the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences center. Cells were cultured in DMEM (GIBCO, Invitrogen, Waltham, MA, USA; penicillin 100 U/mL, streptomycin 100 µg/mL) with 10% fetal bovine serum at the condition of 37°C, 5% CO₂. Cells in the logarithmic growth phase were used for experiments.

Soft agar clone formation experiment

The bottom of six-well plates was covered with 1.5 mL of agar (DMEM medium containing 10% FBS and 0.5% Difco agar noble). The CCAT1-L-specific siRNAs (lncRNA siRNA #1 and lncRNA siRNA #3, as shown in Figure 1), as well as noncoding siRNA (control) were used to transfect human gastric adenocarcinoma cell lines MGC803 and MKN-28 seeded at the density of 2.5×10^3 /mL in the upper agar (DMEM medium containing 10% FBS and 0.35% Difco agar noble). The cells were cultured for 2 weeks in 37°C and

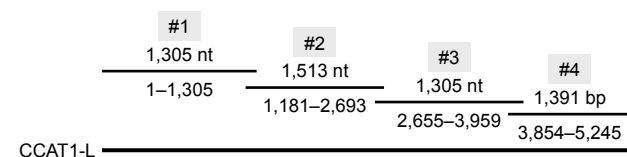


Figure 1 Design of CCAT1-L siRNAs.

Abbreviation: CCAT1-L, colorectal cancer-associated transcript 1-long.

5% CO₂. After visible colonies were formed, the cells were stained with 0.5% crystal violet (Soleil, Beijing, China).

Scratch wound assay

The cells were harvested with 0.25% trypsin-EDTA solution (Sigma, Ronkonkoma, NY, USA). The precipitated cells were centrifuged (5,000 rpm, 5 minutes) in a 15 mL tube. The supernatant was aspirated, and the cells were resuspended in culture medium. One million cells were seeded in six-well plates. After 24 hours, cells reached 100% confluence. Scratch was made using a 10 µL pipette in a sterile environment. PBS was used to wash the plates for two times to remove floating cells. Serum-free medium was then added. The plates were then placed in 37°C and 5% CO₂. Photographs of the plate were taken at 0 and 48 hours after scratches were made.

Transwell assay

Cells were harvested using 0.25% trypsin-EDTA. The solution was then centrifuged and cells were resuspended in a serum-free media containing 0.1% BSA. Six hundred microliters of complete medium containing 10% FBS was then added to the lower layer of Transwell 24-well plates (Millipore, Burlington, MA, USA). Cell suspension in the upper layer (200 µL, 4×10⁵ cells) was incubated at 37°C and 5% CO₂. Migration and invasion were assessed after 24 and 50 hours, respectively, by fixing the cells with methanol for 30 minutes and staining the cells with 0.1% crystal violet for 15 minutes.

Animal handling

Twenty female BALB/C-(nu/nu) nude mice, weighing 18±2 g, 4–6 weeks of age, were purchased from Jinan Banyue Experimental Animal Breeding Co. Ltd. (Animal Production License No SCXK 20140007) and randomly divided into two groups (N=10 for each group). The mice were housed in an environment maintained at a temperature of 25°C±5°C, average humidity 55%±5%, 12 hours of light and dark cycle. The study was approved by the institutional animal care committee of The Affiliated Yantai Yuhuangding Hospital of Qingdao University.

Nude mice were divided into two groups (n=20). The tumor cells MKN-28 transfected with CCAT-L siRNA #1 or control siRNA (1×10⁷ cells in 100 µL Matrigel/PBS mixture) were subcutaneously implanted into nude mice ribs on both sides. Ten nude mice were randomly selected from each group for survival analysis. Five days after inoculation, the survival of mice was observed daily. The tumor size was measured every 2–3 days by calipers. The average volume of tumor in each group was calculated: volume

(mm³) = (length × width²)/2. Tumors were surgically removed and weighed at 20 days after tumor inoculation. For a total time of 80 days, we documented the death of mice at different time points, calculated survival rate, and plotted survival curve. Anesthesia was performed by intraperitoneal injection of 5% pentobarbital at a dose of 50 mg/kg and the mice were sacrificed by cervical dislocation.

Western blot

Gastric adenocarcinoma tissue and adjacent normal tissues were homogenized and centrifuged to extract proteins. After washing with PBS, cells were lysed by sonication, centrifuged at 13,000 rpm for 30 minutes at 4°C. Protein concentration was determined by BCA assay (Biorad, Hercules, CA, USA). Proteins were then resolved by SDS-PAGE and transferred to polyvinylidene fluoride membrane (Merck, Darmstadt, Germany). The membrane was then blocked with TBST solution containing 5% nonfat dry milk for 1 hour. Rabbit polyclonal primary antibodies against MYC (1:1,000, ABIN969300; antibodies-online, Pleasanton, CA, USA), RAS (1:1,000, ABIN2485499; antibodies-online), T-ERK (1:200, MA1055; Boster, Pleasanton, CA, USA), P-ERK (1:1,000, ABIN744713; Bioss, Freiburg, Germany), E-cadherin (1:100, ABIN1387847; Bioss), and vimentin (1:200, ABIN1686955; Spring Bioscience, Pleasanton, CA, USA) were used. After incubating the membranes at 4°C overnight, the membranes were incubated with horseradish peroxidase-labeled goat anti-rabbit IgG (1:1,000; Proteintech, Rosemont, IL, USA) for 1 hour. The signal on the membrane was detected by enhanced chemiluminescence. The protein expression level was normalized by GAPDH. The membranes were then scanned. Quantification of the protein content was performed using ImageJ software (National Institutes of Health, Rockville Pike, Bethesda, MD, USA).

Statistical methods

SPSS 19.0 statistical software was used to analyze data. The data were expressed as mean ± SD, and Student's *t*-test was performed to compare the data between two groups. The one-way ANOVA was used to compare the data between multiple groups. Follow-up data were analyzed using the least significant difference test, with *P*<0.05 indicating statistically significant differences.

Results

The expression of long noncoding RNA CCAT1-L mRNA and c-MYC mRNA

To validate the role of CCAT1-L as a biomarker in gastric adenocarcinoma, we compared the expression of CCAT1-L

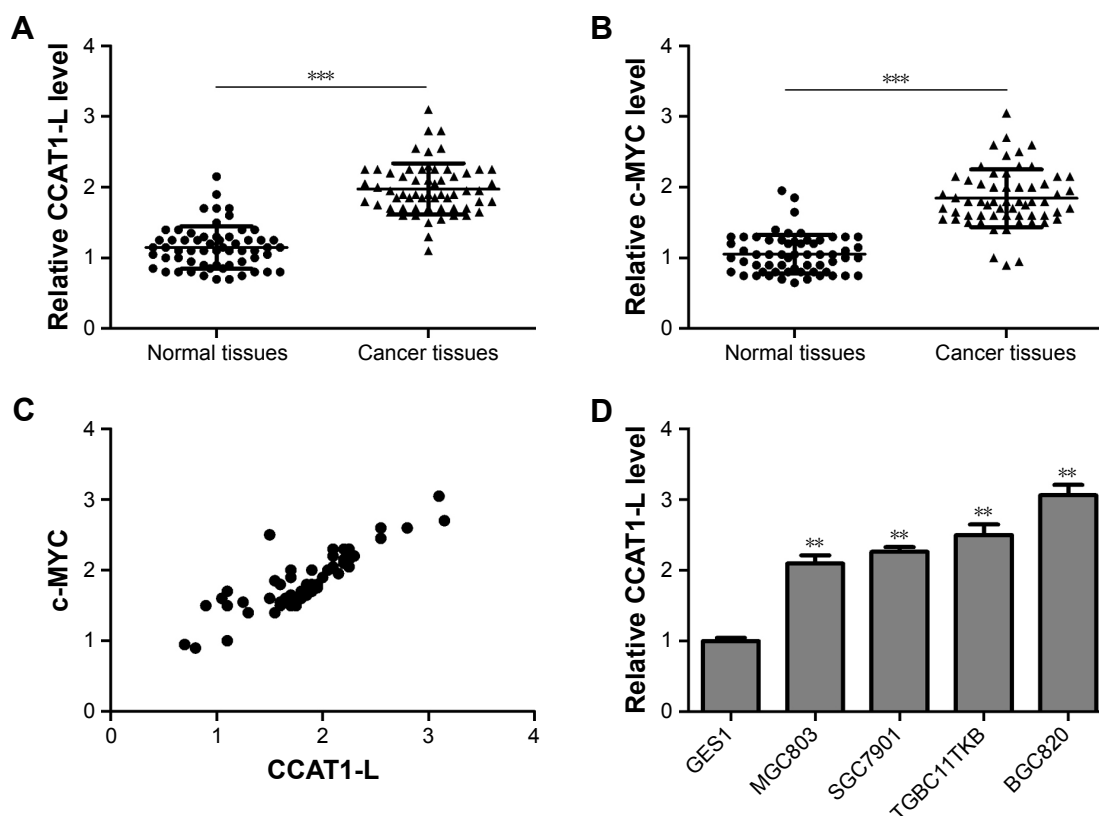


Figure 2 The expression of CCAT1-L mRNA and c-MYC mRNA.

Notes: (A) qRT-PCR detection of CCAT1-L expression in gastric adenocarcinoma and adjacent normal tissue ($n=60$; $***P<0.001$). (B) qRT-PCR detection of c-MYC expression in gastric adenocarcinoma and adjacent normal tissue ($n=60$; $***P<0.001$). (C) The correlation between the expression level of CCAT1-L mRNA and c-MYC mRNA ($r=0.863$, $P<0.001$). (D) qRT-PCR detection of CCAT1-L expression in gastric adenocarcinoma cell lines ($n=3$; $**P<0.01$ vs CCAT1-L expression in GES1 cells).

Abbreviations: CCAT1-L, colorectal cancer-associated transcript 1-long; qRT-PCR, quantitative real-time polymerase chain reaction.

in gastric cancer tissues and cells. qRT-PCR showed that the expression of CCAT1-L in tumor tissues was significantly higher than that in adjacent normal tissues ($P<0.001$; Figure 2A). The expression of c-MYC mRNA in tumor tissues was significantly higher than that in adjacent normal tissues ($P<0.001$; Figure 2B). As shown in Figure 2C, there was a positive correlation between the expression level of CCAT1-L mRNA and c-MYC mRNA ($r=0.863$, $P<0.001$). Further, CCAT1-L expression in four different gastric adenocarcinoma cell lines, MGC803, MKN-28, SGC7901, and BGC823, was also markedly higher than that in normal gastric cell line, GES1 (Figure 2D; $n=3$; $P<0.01$). These data corroborated that CCAT1-L upregulation was closely associated with gastric adenocarcinoma, suggesting that regulating the level of CCAT1-L may be a strategy to impede the development of this highly lethal disease.

CCAT1-L knockout significantly inhibits the proliferation of gastric adenocarcinoma cells

To verify the antitumor efficacy of CCAT1-L knockdown in gastric adenocarcinoma, CCAT1-L-specific siRNA was

transfected into gastric adenocarcinoma cell lines, followed by monitoring cell migration and invasion. The design of CCAT1-L siRNAs is shown in Figure 1. Two siRNAs, siRNA #1 and siRNA #3 were primarily used in the present study. As shown in Figure 3A, lncRNA siRNA #1 and lncRNA siRNA #3 were able to pronouncedly reduce the expression level of CCAT1-L in tumor cells ($P<0.001$), while control siRNA did not induce significant change in CCAT1-L expression ($P>0.05$). Therefore, cells transfected with control siRNA were used as the control group in further studies. CCAT1-L knockdown in gastric adenocarcinoma cell lines, MGC803 and MKN-28, resulted in a dramatic reduction in the number of colonies formed ($P<0.001$; Figure 3B and C, $n=3$), suggesting dampened cell proliferation and migration. Since CCAT1-L expression of MKN-28 is more pronounced than that of MGC803 and the tumor-inhibitory effect of lncRNA siRNA #1 was stronger than that of lncRNA siRNA #3, MKN-28 cells transfected with CCAT1-L siRNA #1 were further used to establish tumor xenografts to verify whether CCAT1-L knockdown suppresses tumor growth in vivo. As expected, CCAT1-L knockdown significantly inhibited tumor growth in nude mice

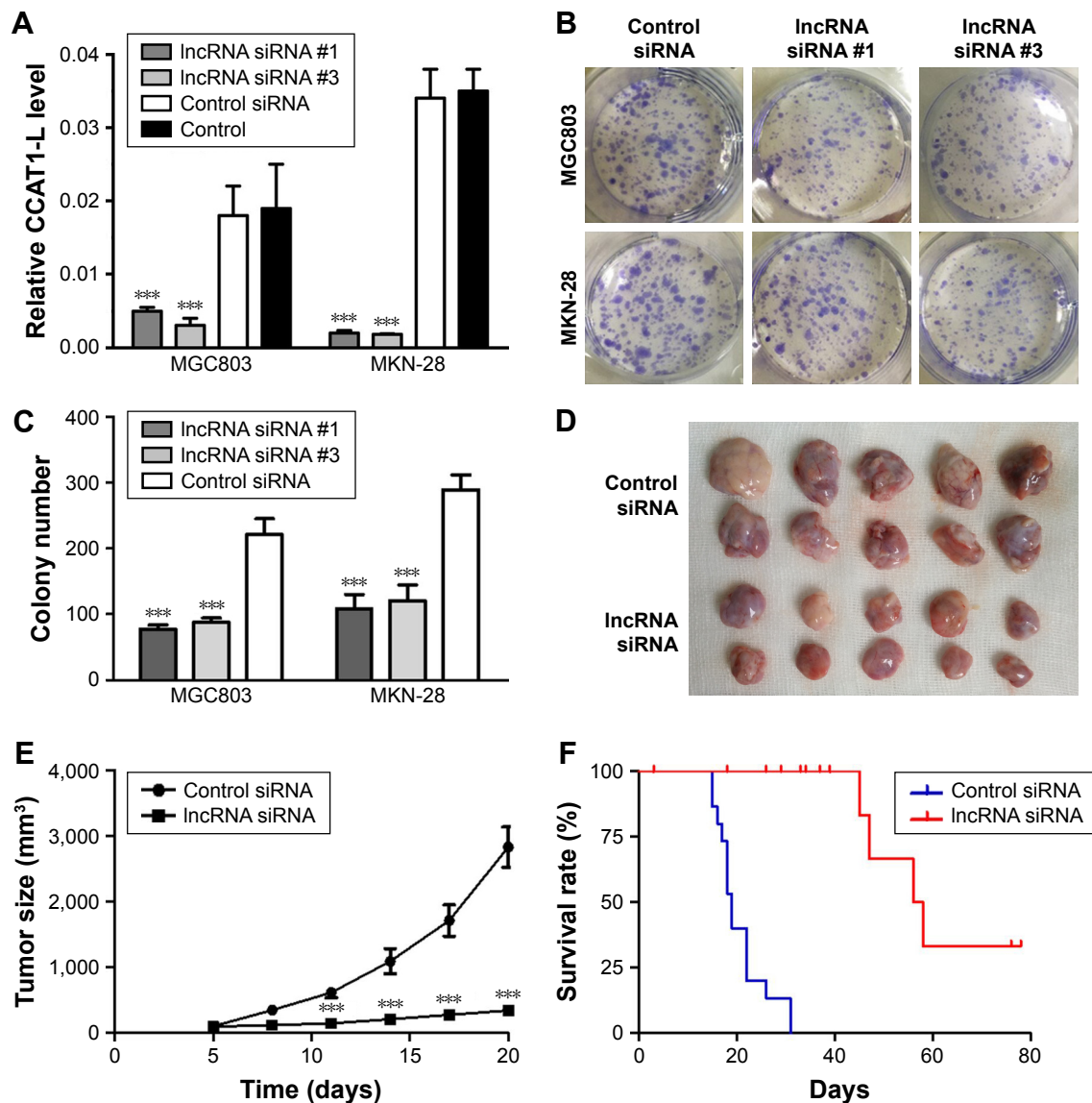


Figure 3 CCAT1-L knockdown effectively inhibited tumor growth both in vitro and in vivo.

Notes: (A) Verification of CCAT1-L knockdown by specific siRNA (CCAT1-L siRNA #1 and CCAT1-L siRNA #3) and control siRNA using qRT-PCR (n=3). (B, C) Colony formation analysis of cells after siRNA transfection. (D) Picture of MKN-28 tumors harvested from different treatment groups. (E) In vivo tumor growth of MKN-28 cells transfected with CCAT1-L siRNA #1 or control siRNA. (F) Survival curve of mice bearing MKN-28 tumors transfected with CCAT1-L siRNA or control siRNA (n=10). *** $P < 0.01$ vs control siRNA.

Abbreviations: CCAT1-L, colorectal cancer-associated transcript 1-long; qRT-PCR, quantitative real-time polymerase chain reaction.

($P < 0.001$; Figure 3D, n=10, Figure 3E, n=2). In addition, almost all of the mice without CCAT1-L knockdown died after 22 days. In contrast, >50% of mice with CCAT1-L knockdown survived after 22 days (Figure 3F, n=10). These results indicate that lncRNA CCAT1-L knockout can effectively inhibit tumor growth and proliferation in vivo.

CCAT1-L knockdown significantly inhibits gastric adenocarcinoma cell migration and invasion

Scratch wound assay indicated that CCAT1-L knockdown significantly inhibited gastric adenocarcinoma cell migration (35.0%; n=3; $P < 0.01$) compared with the control siRNA

group (Figure 4A and B). Transwell assay also confirmed that CCAT1-L knockdown significantly inhibited metastasis and invasiveness of gastric adenocarcinoma cells (54.8% and 57.9%, respectively; n=3; $P < 0.01$; Figure 4C and D). These data indicated that the inhibition of CCAT1-L can effectively inhibit the migration and invasion abilities of gastric adenocarcinoma cells.

CCAT1-L promotes the proliferation and metastasis of tumor cells by inducing MYC expression

We next examined the mechanism of CCAT1-L knockdown in regulating gastric adenocarcinoma progression.

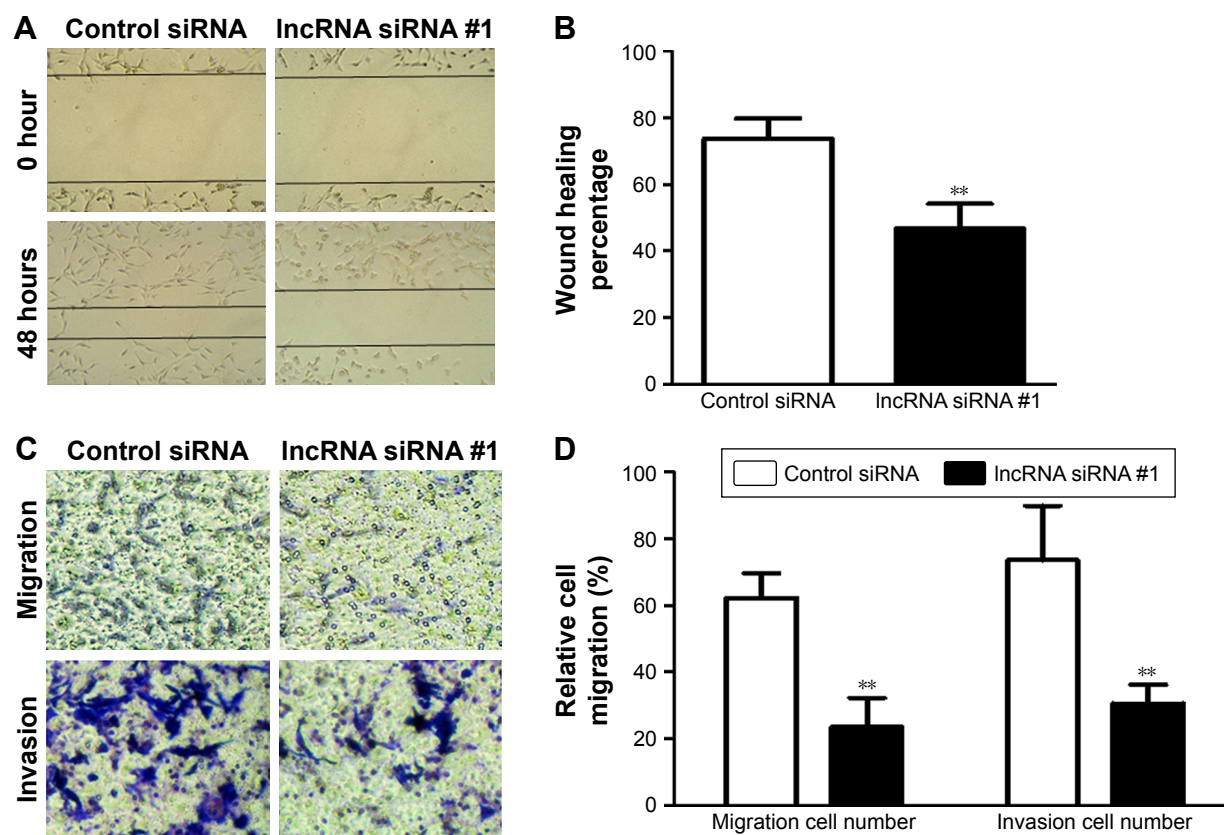


Figure 4 Inhibition of CCAT1-L expression effectively inhibits gastric tumor cell migration and invasion.

Notes: (A, B) Representative images and quantification of scratch wound assay gastric adenocarcinoma cell migration ($n=3$, $**P<0.01$). (C, D) Images and quantification of transwell assay of gastric adenocarcinoma cells ($n=3$, $**P<0.01$). Magnification $\times 200$.

Abbreviation: CCAT1-L, colorectal cancer-associated transcript 1-long.

Considering the close alliance of CCAT1-L and MYC protein, we explored whether the expression of MYC protein in gastric adenocarcinoma samples and gastric adenocarcinoma cells was affected by CCAT1-L knockdown. In patient gastric adenocarcinoma tissues, the expression of MYC was also significantly higher than that in adjacent normal tissues (Figure 5A). Inhibition of CCAT1-L in gastric adenocarcinoma cell line MKN-28 significantly reduced the level of MYC protein, with a more significant decrease in MYC protein after transfection of siRNA #1 (Figure 5B). This result suggested that CCAT1-L knockdown mediated proliferation and invasion of gastric adenocarcinoma cells through the regulation of MYC protein. To further elucidate this process, we examined the changes of EMT-related proteins after CCAT1-L knockdown by Western blot. Our study showed that knockdown of CCAT1-L significantly inhibited the expression of RAS, vimentin, and the phosphorylation of ERK protein in gastric adenocarcinoma cell line MKN-28; meanwhile, the expression of E-cadherin protein was significantly increased (Figure 5C). In conclusion, CCAT1-L may promote the proliferation, migration, and invasion of tumor

cells by regulating the expression of MYC, which activates the expression of EMT-related proteins.

Discussion

Cancer biomarkers research is paramount to the development of molecules that facilitate accurate diagnosis and efficient treatment of a variety of cancers. lncRNAs are an emerging class of cancer biomarkers that are increasingly used in cancer diagnosis and therapy.^{13–16} Previously, we discovered the CCAT1-L, an lncRNA abundantly transcribed from the upstream region of MYC. CCAT1-L was found to be highly expressed in colorectal cancer as a cancer enhancer.¹⁰ The important role of CCAT1-L as a therapeutic target also revealed that knockdown of CCAT1-L successfully impeded the progression of colorectal cancer.¹⁰ Here we broadened the clinical importance of CCAT1-L by showing that CCAT1-L is highly expressed in gastric adenocarcinoma and knockdown of CCAT1-L inhibited the proliferation, migration, and invasion of gastric adenocarcinoma. We first showed that CCAT1-L is upregulated in gastric adenocarcinoma samples compared with healthy

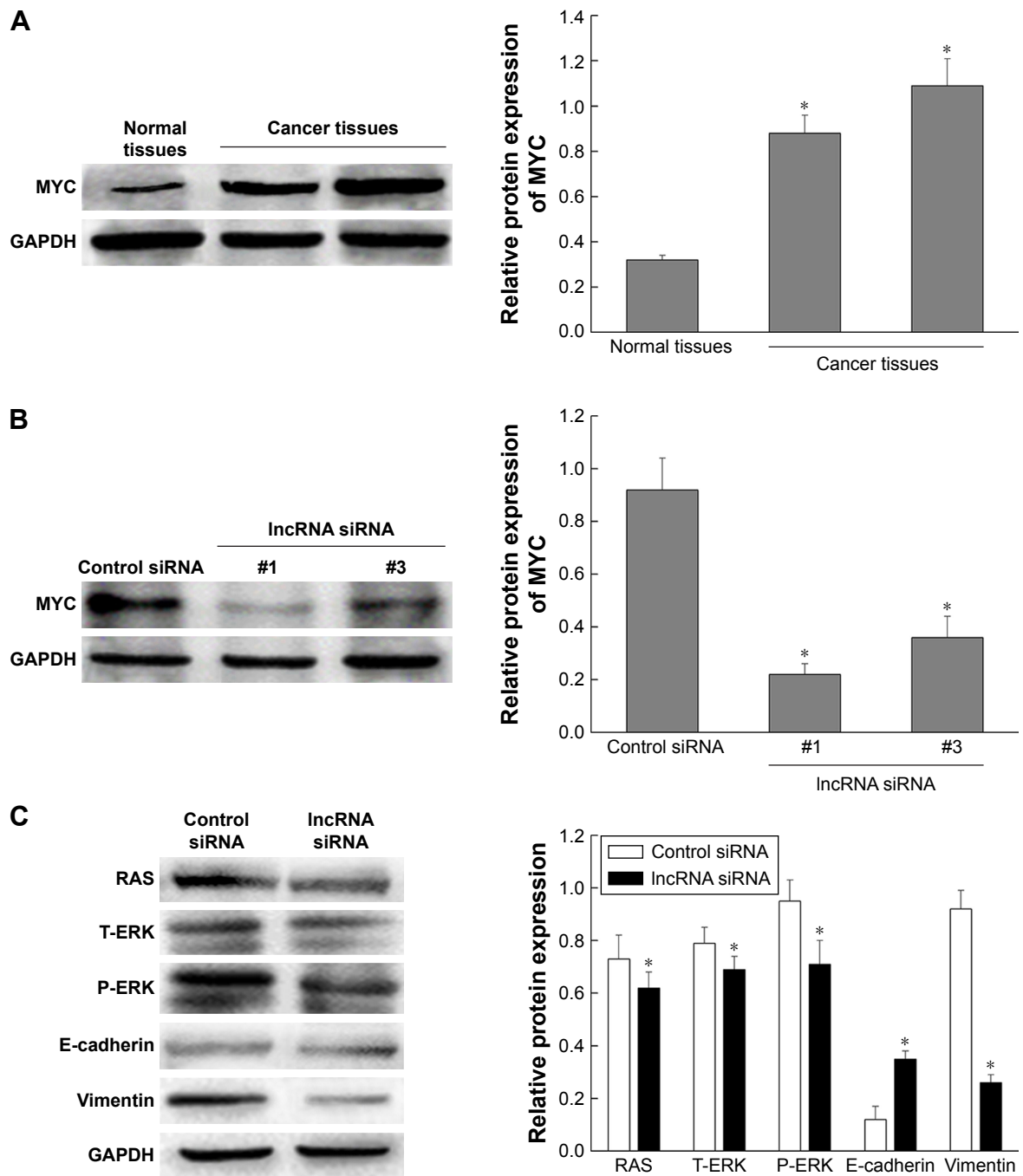


Figure 5 CCAT1-L promotes MYC expression in tumor cells.

Notes: (A) Western blot analysis of MYC protein expression in two clinical gastric adenocarcinoma tumor samples and one adjacent normal tissue sample. (B) Western blot analysis of MYC protein expression in gastric adenocarcinoma cells after CCAT1-L siRNA transfection with siRNA #1 and #3. (C) Western blot analysis of EMT-related protein expression in gastric adenocarcinoma cell line MKN-28. * $P < 0.01$ vs control siRNA.

Abbreviations: CCAT1-L, colorectal cancer-associated transcript 1-long; EMT, epithelial–mesenchymal transition; siRNA, small-interfering RNA.

stomach samples in patients. In gastric cancer cells, we also observed an upregulation of CCAT1-L. These results resonate with previous evidences that lncRNA CCAT1 is highly expressed in gastric adenocarcinomas.² In this study, we did not explore the correlation of CCAT1-L and tumor stages. Further studies are warranted to elucidate this correlation to

strengthen the clinical utility of CCAT1-L as a biomarker in gastric adenocarcinoma.

In accordance with the fact that CCAT1-L upregulation is associated with gastric adenocarcinoma, we explored if knockdown of CCAT1-L is able to inhibit gastric cancer development. RNA interference was used to suppress CCAT1-L

expression. As expected, CCAT1-L knockdown prominently reduced the tumor growth and improved survival of the mouse models. Consistently, in vitro cell study demonstrated that CCAT1-L knockdown suppressed gastric cancer migration and invasion in the scratch wound assay and transwell migration study. This serves as clear evidence that CCAT1-L knockdown is an effective strategy to suppress gastric cancer progression. Notably, the in vivo study used tumor cells already transfected with CCAT1-L siRNAs. Although the effect of siRNA is relatively transient,¹⁷ the antitumor efficacy is sufficiently prominent. To establish an approach that is more clinically translatable, it is imperative to transfect siRNAs in vivo in tumor cells. This demands specific delivery of siRNAs to tumor cells cytoplasm for effective action. Given recent advances in gene delivery strategies^{18,19} and the potent antitumor efficiency of CCAT1-L knockdown approach, it can be envisioned that CCAT1-L can be considered a potential tool for gastric cancer treatment.

Since CCAT1-L is transcribed from the upstream region of MYC, which is one of the key factors in EMT, we also explored if CCAT1-L knockdown affects EMT of gastric cancer cells. Despite that EMT may adopt cancer-promoting or cancer-inhibiting roles at different stages of cancer,²⁰ it is widely recognized that cells that have undergone EMT are characterized by higher metastatic potential, leading to poorer survival.²¹ We demonstrated that CCAT1-L knockdown significantly reduced the expression of RAS, vimentin, and phosphorylated ERK, while increasing E-cadherin expression. These data indicated that CCAT1-L knockdown dampened EMT, which also explained the antitumor effect of CCAT1-L knockdown.

Conclusion

lncRNA CCAT1-L and MYC are overexpressed in gastric adenocarcinoma. When CCAT1-L was knocked down, the proliferation, migration, and invasion abilities of gastric adenocarcinoma cells were decreased, and the mortality of mice with gastric adenocarcinoma cell line was also decreased. At the same time, the activity of epithelial–mesenchymal transition protein was also decreased. Therefore, the inhibition of CCAT1-L expression can inhibit the epithelial–mesenchymal transition of gastric adenocarcinoma cells and thus inhibits the metastasis of gastric adenocarcinoma, which has a positive effect in preventing the metastasis and treatment of gastric adenocarcinoma.

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Disclosure

The authors report no conflicts of interest in this work.

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