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ORIGINAL RESEARCH

Long intergenic noncoding RNA 00707 promotes colorectal cancer cell proliferation and metastasis by sponging miR-206

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Background: The incidence and mortality of colorectal cancer (CRC) are rising worldwide. Long-noncoding RNAs (lncRNAs) are known to play key roles in the development of human cancers, including CRC. However, the function and underlying mechanism of long intergenic noncoding RNA 00707 (LINC00707) in the development of CRC are unknown.

Materials and methods: The expression of LINC00707 and miR-206 in tissue samples or cell lines was measured by quantitative reverse transcription PCR (qRT-PCR). The protein expression of neurogenic locus notch homolog protein 3 (NOTCH3) and transmembrane 4 L6 family member 1 (TM4SF1) was assessed by Western blotting. Cell proliferation, migration, and invasion were assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and transwell assays. Luciferase reporter assay and biotin-coupled miRNA capture assay were used to explore the relationship between LINC00707 and miR-206 expression.

Results: The expression of LINC00707 was significantly upregulated in CRC tissues as compared with the adjacent non-CRC tissues. LINC00707 expression was significantly correlated with tumor size, lymphatic metastasis, and distant metastasis, but not significantly correlated with age and gender. Knockdown of LINC00707 expression significantly inhibited LoVo and HCT116 cell proliferation, migration, and invasion. LINC00707 acted as a molecular sponge by competing for miR-206 and indirectly modulating the expression of its targets, NOTCH3 and TM4SF1.

Conclusion: LINC00707 promotes CRC cell proliferation and metastasis by sponging miR-206, suggestive of its potential application for CRC treatment.

Keywords: long intergenic noncoding RNA 00707, colorectal cancer, miR-206, NOTCH3, TM4SF1

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed malignancy and the second leading cause of cancer-related mortality worldwide.[1] CRC incidence and associated mortality rates are gradually rising in China.[2] Despite tremendous efforts for improvement in the clinical treatment of CRC, the 5-year overall survival rate of CRC is unsatisfied owing to cancer recurrence and metastasis.[3] Therefore, elucidation of the molecular mechanism underlying the progression of CRC is desirable for the development of new treatment strategies and improvement of patient prognosis.

Long-noncoding RNAs (lncRNAs) are transcripts that are more than 200 nucleotides in length without or with limited protein-coding capacity.[4–6] LncRNAs play

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essential roles in several biological processes, including embryonic development, cell growth and differentiation, and tumorigenesis.[7] Therefore, lncRNAs may contribute to the development and progression of various human diseases, including cancers.[7–10] Long intergenic noncoding RNA 00707 (LINC00707) is a 3,087-bp noncoding RNA located on chromosome 10p14. The knockdown of LINC00707 expression was shown to repress lung adenocarcinoma cell proliferation and migration.[11] Wang et al found that LINC00707 promotes hepatocellular carcinoma (HCC) progression.[12] However, the function and underlying mechanism of LINC00707 in the development of CRC remain to be elucidated.

Although the knowledge on the molecular mechanisms of lncRNAs still remains limited, more and more reports reveal that LncRNAs function as competing endogenous RNAs (ceRNAs) or natural microRNA (miRNA) sponges, thereby relieving the repression of target mRNAs.[13] miRNAs are small non-coding RNAs within a size range of 21–25 nucleotides, repress the translation of their target mRNAs or degrade mRNAs.[14] However, whether LINC00707 functions as ceRNA of miRNAs to regulate tumor progression in CRC remains to be investigated.

In the present study, the expression of LINC00707 in CRC tissues was measured and the relationship between LINC00707 expression and clinicopathologic characteristics of CRC patients was analyzed. Functional effects of LINC00707 on CRC cell growth and metastasis were assessed in vitro. Moreover, the underlying mechanism of LINC00707 functions in CRC was investigated by confirming whether LINC00707 functions as ceRNA of miR-206 to thereby relieve the translation repression of target mRNA, neurogenic locus notch homolog protein 3 (NOTCH3) and transmembrane 4 L6 family member 1 (TM4SF1).[15,16]

Materials and methods

Clinical tissue samples

A total of 40 CRC tissues and paired adjacent tissue samples (at least 5 cm away from the tumor tissues) were collected by surgical resection from patients with CRC who had received neither chemotherapy nor radiotherapy at the Third Affiliated Hospital of Xinxiang Medical University Hospital. The procedures of this study were approved by the Ethics Committee of Third Affiliated Hospital of Xinxiang Medical University. Written informed consent was received from all participants in accordance with the Declaration of Helsinki. The diagnosis and clinicopathological characteristics included age, gender, tumor size, lymph node metastasis, and distant metastasis, which were confirmed by two pathologists (Table S1). All the tissue samples were immediately frozen in liquid nitrogen and stored at −80°C until RNA extraction.

Quantitative reverse transcription PCR (qRT-PCR)

Total RNA from tissues or cultured cells was separated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA (1 μg) was reverse transcribed to cDNA with reverse transcription kit (Takara, Dalian, China). qRT-PCR was performed on ABI 7500 RT-PCR system (Applied Biosystems, Foster City, CA, USA) with SYBR® Premix Ex Taq™ Kit (TaKaRa) and Mir-X™ miRNA qRT-PCR SYBR® Kit (Clontech Laboratories, Inc., Mountain View, CA, USA) in accordance with the manufacturer's protocol. The primers were synthesized by GenePharma (Shanghai, China) and the primer sequences were as follows: LINC00707 forward 5′-CCAACAGGGTATCAGAATTCTC-3′ and reverse 5′-TGCTGACAATAGCCATTAGG-3′; β-actin forward 5′-TGGATCAGCAAGCA GGAGTA-3′ and reverse 5′-TCGGCCACATTGTGAACTTT-3′; miR-206 forward, 5′-CAGATCCGATTGGAATGTAAGG-3′ and reverse, 5′-TATGCTTGTTCTCGTCTCTGTGTC-3′; U6 forward 5′-CTCGCTTCGGCAGCACA-3′ and reverse 5′-AACGCT TCACGAATTTGCGT-3′. The expression of LINC00707 and miR-206 was normalized to the expression of β-actin and U6, respectively. Data were analyzed by comparative cycle threshold (CT) ($2^{-\Delta\Delta CT}$) method. All reactions were performed in triplicates.

Cell culture and transfection

The human CRC cell lines LoVo and HCT116 were purchased from the Shanghai Institutes of Biological Sciences (Shanghai, China). Cells were maintained in Dulbecco's modified Eagle medium (DMEM; HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; HyClone) and 1% penicillin-streptomycin (Thermo Scientific, Waltham, MA, USA) and cultivated at 37°C in 5% CO_2. LINC00707 siRNA (si-LINC00707 $1^{\#}$: 5′-GGCUUUCCAUGACCCAUAAUU-3′, si-LINC 00707 $2^{\#}$: 5′-GGAAGCCACU CCUGCAUUUUU-3′, si-LINC00707 $3^{\#}$: 5′-GCAGGAACAUCACCAUCUU UU-3′), siRNA negative control (si-NC), miR-206 mimic, and negative control (NC) were all purchased from GenePharma (Shanghai, China). Lipofectamine™

2000 (Invitrogen) was used for cell transfection according to the manufacturer's instructions.

Cell proliferation assay

Cell proliferation was determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, 2×10^3 cells were seeded in each well of a 96-well plate in 100-μL volume in triplicates. At 24, 48, and 72 hrs after transfection, MTT (10 μL, 5 mg/mL) was added into each well and incubated for 4 hrs at 37°C. After incubation, 100 μL of dimethyl sulfoxide (DMSO) was added to solubilize the formazan. The absorbance was measured at 570 nm wavelength with a microplate reader (Bio-Rad, Hercules, CA, USA).

Transwell migration and invasion assay

Cell migration and invasion were evaluated using the BD 24-well Transwell chamber (BD Biosciences, San Jose, CA, USA). Transfected cells (1×10^5) were seeded in serum-free medium in the top chamber of each well. Matrigel (BD Biosciences) was used to cover the top side of the membrane for invasion assay and Matrigel-free condition was used for migration assay. The lower chamber was filled with complete medium supplemented with 10% FBS. The cells were incubated at 37°C with 5% CO_2 for 48 hrs, and the non-traversed cells were removed from the upper filter with a cotton swab. Next, the ratio of cells for migration and invasion assays was determined using two methods, crystal violet staining and MTT assay. For crystal violet staining, the cells that migrated or invaded to the reverse side of chamber inserts were fixed with methanol for 30 mins and stained with crystal violet for 15 mins. The number of migrated or invasive cells were determined by counting the stained cells in ten randomly selected fields under a microscope (Olympus, Tokyo, Japan). For MTT assay, 500 μL complete medium containing 0.5 mg/mL MTT was added into the lower chamber of each well. The top chamber was immersed in the medium and incubated for 4 hrs at 37°C. After incubation, 100 μL of DMSO was added to solubilize the formazan. The absorbance was measured at 570 nm wavelength with a microplate reader (Bio-Rad, Hercules, CA, USA). All experiments were performed in triplicates.

Western blot analysis

Cellular proteins were extracted with a lysis buffer supplemented with a protease inhibitor phenylmethanesulfonyl fluoride (PMSF; Beyotime, Shanghai, China). Protein concentration was determined with the bicinchoninic acid (BCA) protein assay kit (Beyotime). Equivalent amounts of denatured proteins (20 μg) were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). After blocking, the membranes were incubated at 4°C overnight with following primary antibodies: anti-NOTCH3 (dilution 1:1,000, Cell Signaling Technology), anti-TM4SF1 (dilution 1:1,000, Thermo Scientific), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (dilution 1:5,000; Abcam). After washing with Tris-buffered saline containing Tween-20 (TBST), the membrane was incubated with a horseradish peroxidase (HRP)-labeled secondary antibody (dilution 1:5,000, Boster, Wuhan, China) for 2 hrs at 25°C, followed by washing with TBST. The immunolabeled proteins were reacted with chemiluminescent HRP substrate (Thermo Scientific, Waltham, MA, USA).

Bioinformatic analysis

The potential binding sites of miRNAs on LINC00707 were predicated using lncRNABase (http://starbase.sysu.edu.cn/agoClipRNA.php?source=lncRNA).

Luciferase reporter assay

LINC00707 wild-type (WT) with potential miR-206-binding sites or mutant (MUT) of each sites were generated and fused to a luciferase reporter vector psi-CHECK-2 (Promega, Madison, WI, USA) to analyze the interaction between LINC00707 and miR-206. The binding site of miR-206 on LINC00707 was mutated into AGATAGGA. HEK293T cells were co-transfected with luciferase plasmids and miR-206 mimic or miRNA negative control. After 48 hrs transfection, luciferase activities were detected with a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. The ratio of firefly to *Renilla* luciferase activity was assessed.

Biotin-coupled miRNA capture assay

The biotin-coupled miRNA capture assay was performed as previously described.[17] In brief, miR-206 mimics and miRNA negative control were labeled with biotin at the 3′ end and transiently transfected into LoVo and HCT116 cells. After 24 hrs of culture, the cells were harvested and lysed in a lysis buffer, followed by incubation with streptavidin beads (Thermo Scientific) to pull-down the biotin-coupled RNA complex. After washing with lysis buffer, TRIzol reagent (Invitrogen) was used to recover the RNAs that

specifically interacted with miRNA. The abundance of LINC00707 in the bound fraction was measured with qRT-PCR after its reverse transcription to cDNA.

Statistical analysis

All statistical analyses were performed using the Statistical Package for the Social Sciences version 19.0 software (SPSS Inc., Chicago, IL, USA). All data are expressed as the mean±standard deviation (SD). Student's *t*-test and one-way analysis of variance were carried out to evaluate significant differences. Statistical significance was considered at $P<0.05$.

Results

LINC00707 expression is upregulated in CRC tissues

The expression of LINC00707 in 40 CRC tissues and paired adjacent non-CRC tissues was detected with qRT-PCR analysis (Figure 1). The expression of LINC00707 was significantly upregulated in CRC tissues as compared with the adjacent non-CRC tissues.

LINC00707 expression correlates with clinicopathologic characteristics of patients with CRC

The relationship between various clinicopathological characteristics of patients with CRC and LINC00707 expression was analyzed (Figure 2). Age and gender did not show significant correlation with LINC00707 expression, while tumor size, lymphatic metastasis, and distant metastasis showed significant correlation with LINC00707 expression.

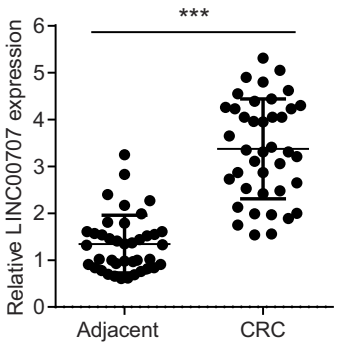

Figure 1 Expression of LINC00707 in colorectal cancer (CRC) tissues and paired adjacent non-colorectal cancer tissues was detected by quantitative reverse transcription PCR analysis (***$P<0.001$).

Knockdown of LINC00707 inhibits cell proliferation, migration, and invasion in LoVo and HCT116 cells

To investigate whether LINC00707 expression has functional impacts on CRC cells, the expression of LINC00707 in two CRC cell lines (LoVo and HCT116) was silenced by transfection with LINC00707 siRNA (si-LINC00707). qRT-PCR results showed that the expression of LINC00707 in both LoVo and HCT116 cells was downregulated upon transfection with si-LINC00707, and the silencing effect of si-LINC00707 $3^{\#}$ was significantly higher than that of si-LINC00707 $1^{\#}$ and si-LINC00707 $2^{\#}$ (Figure 3A). Thus, si-LINC00707 $3^{\#}$ was selected for further functional assays.

The effects of LINC00707 knockdown on LoVo and HCT116 cells were investigated. MTT and transwell assay results demonstrate that LINC00707 knockdown significantly inhibited the proliferation (Figure 3B and C), migration (Figure 3D and E), and invasion (Figure 3F and G) of both LoVo and HCT116 cells.

LINC00707 acts as a molecular sponge for miR-206 and indirectly modulates the expression of its target proteins, NOTCH3 and TM4SF1

LncRNAs have the ability to directly bind to miRNAs and function as a molecular sponge. We investigated whether LINC00707 exerts similar functions. Bioinformatics analysis revealed that the binding sites of miR-206 on LINC00707 sequence are more than other miRNAs, which are downregulated in CRC tissues and suppress tumor progression in CRC cells. So miR-206 was selected for the further study. The putative binding sites between LINC00707 and miR-206 were shown in Figure 4A. The results of the dual-luciferase reporter assay showed that the cells co-transfected with WT LINC00707 and miR-206 mimic exhibited low luciferase activity, while those co-transfected with MUT LINC00707 and miR-206 mimic had no obvious inhibitory effect on luciferase activity (Figure 4B). In addition, biotin-coupled miRNA capture assay results indicated that LINC00707 level was higher in biotin-miR-206 transfected LoVo and HCT116 cells as compared with the cells treated with biotin-miR-NC (Figure 4C). qRT-PCR results showed that the expression level of miR-206 increased in both LoVo and HCT116 cells transfected with si-LINC00707 as compared with those transfected with si-NC (Figure 4D). Western blot

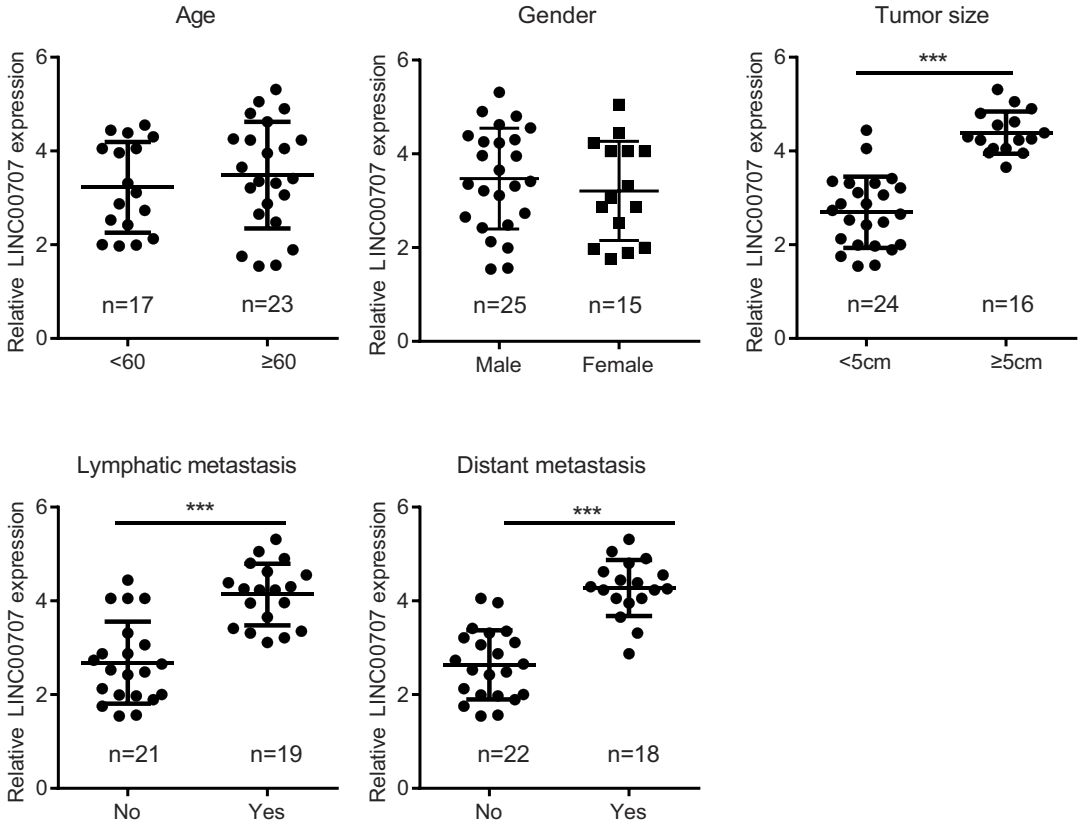

Figure 2 Correlation between LINC00707 expression and clinicopathological characteristics of patients with colorectal cancer. LINC00707 expression was significantly correlated with tumor size, lymphatic metastasis, and distant metastasis, but not significantly correlated with age and gender (***P<0.001).

analysis showed that the protein expression of NOTCH3 and TM4SF1 was downregulated in LoVo and HCT116 cells transfected with si-LINC00707 as compared with the cells transfected with si-NC (Figure 4E).

Discussion

Studies have demonstrated the important role of lncRNAs in the development and progression of various human cancers, including CRC. Therefore, better understanding of the function and underlying molecular mechanism of lncRNAs may help in the discovery of effective therapeutic strategies for the treatment of patients with CRC. In this study, we found that LINC00707 expression level was significantly upregulated in CRC tissues as compared with the adjacent non-CRC tissues. And LINC00707 expression was significantly correlated with tumor size, lymphatic metastasis, and distant metastasis, but not significantly correlated with age and gender. Knockdown of LINC00707 expression resulted in a significant inhibition of LoVo and HCT116 cell proliferation and metastasis. LINC00707 acted as a molecular sponge by competing for miR-206 and indirectly modulating the expression of its target proteins NOTCH3 and TM4SF1.

Recent studies have indicated the abnormal expression of lncRNAs in CRC. For instance, Lv et al reported that the expression of lncRNA ZEB1-AS1 was markedly upregulated in CRC, and ZEB1-AS1 knockdown inhibited CRC cell proliferation.[18] In the present study, we found that LINC00707 expression level was significantly upregulated in CRC tissues as compared with adjacent non-CRC tissues and significantly correlated with tumor size, lymphatic metastasis, and distant metastasis. These results indicate that LINC00707 expression may be involved in the development of CRC. Our present study showed that LINC00707 knockdown significantly inhibited LoVo and HCT116 cell growth, migration, and invasion, suggesting that LINC00707 may serve as a potential target for CRC treatment. Our results were consistent with the function of LINC00707 in other cancers. The expression level of LINC00707 was shown to be significantly upregulated in lung adenocarcinoma tissues as compared with the paired normal tissues, and LINC00707 expression correlated with TNM stage, tumor size, lymphatic metastasis.[11] LINC00707 knockdown resulted in the

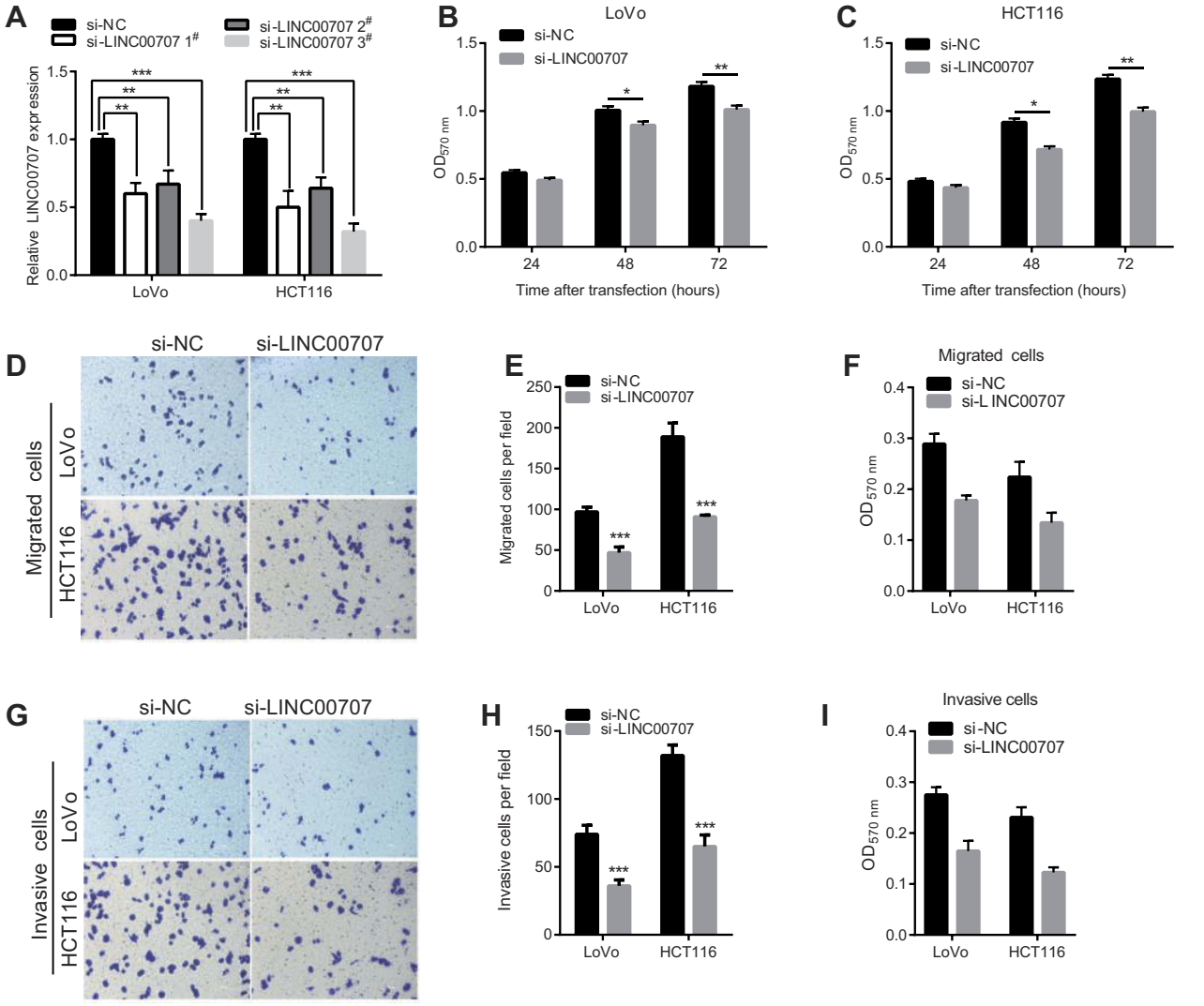

Figure 3 Knockdown of LINC00707 expression inhibits the proliferation, migration, and invasion of colorectal cancer cells. (**A**) Expression level of LINC00707 in LoVo and HCT116 cells transfected with si-LINC00707 1#, si-LINC00707 2#, and si-LINC00707 3#, or si-negative control (NC), as analyzed with quantitative reverse transcription PCR. (**B** and **C**) Knockdown of LINC00707 expression inhibits LoVo (**B**) and HCT116 (**C**) cell proliferation, as evident in the MTT assay. (**D**) The representative graphs of migrated cells stained by crystal violet staining in transwell assay. (**E**) The statistical results of average migrated cells stained by crystal violet in transwell assay. (**F**) The statistical results of $OD_{570\ nm}$ value of migrated cells determined by MTT assay. (**G**) The representative graphs of invasive cells stained by crystal violet staining in transwell assay. (**H**) The statistical results of average invasive cells stained by crystal violet in transwell assay. (**I**) The statistical results of $OD_{570\ nm}$ value of invasive cells determined by MTT assay. *P<0.05, **P<0.01, ***P<0.001.

suppression of lung adenocarcinoma cell proliferation and migration through the regulation of cell division control protein 42 (Cdc42) expression.[11] In HCC, LINC00707 expression was upregulated, and LINC00707 knockdown inhibited the proliferation of cells through the activation of the extracellular signal-regulated kinase/c-Jun N-terminal kinase/protein kinase B signaling pathway.[12]

Recent studies have revealed that lncRNAs act as ceRNAs and interact with miRNAs. LncRNA H19 promotes cell proliferation by sponging miR-200a, thereby increasing β-catenin expression in CRC.[19] LncRNA FTX markedly promoted CRC cell proliferation, migration, and invasion by sponging miR-215.[20] Several studies have reported that miR-206 acts as a tumor suppressor, and its expression was downregulated in various human malignancies.[21,22] In CRC, miRNA-206 levels were significantly downregulated in CRC tissues,[23,24] and miR-206 overexpression inhibited the proliferation, migration, and invasion of CRC cells.[24] The expression profile and function of miR-206 in CRC is opposite to that of LINC00707. So we predicted that LINC00707 may function as ceRNA of miR-206. Here, our study revealed that miR-206 is a target of LINC00707. Changes in LINC00707 expression led to corresponding changes in the expression levels of miR-206, suggestive of the negative

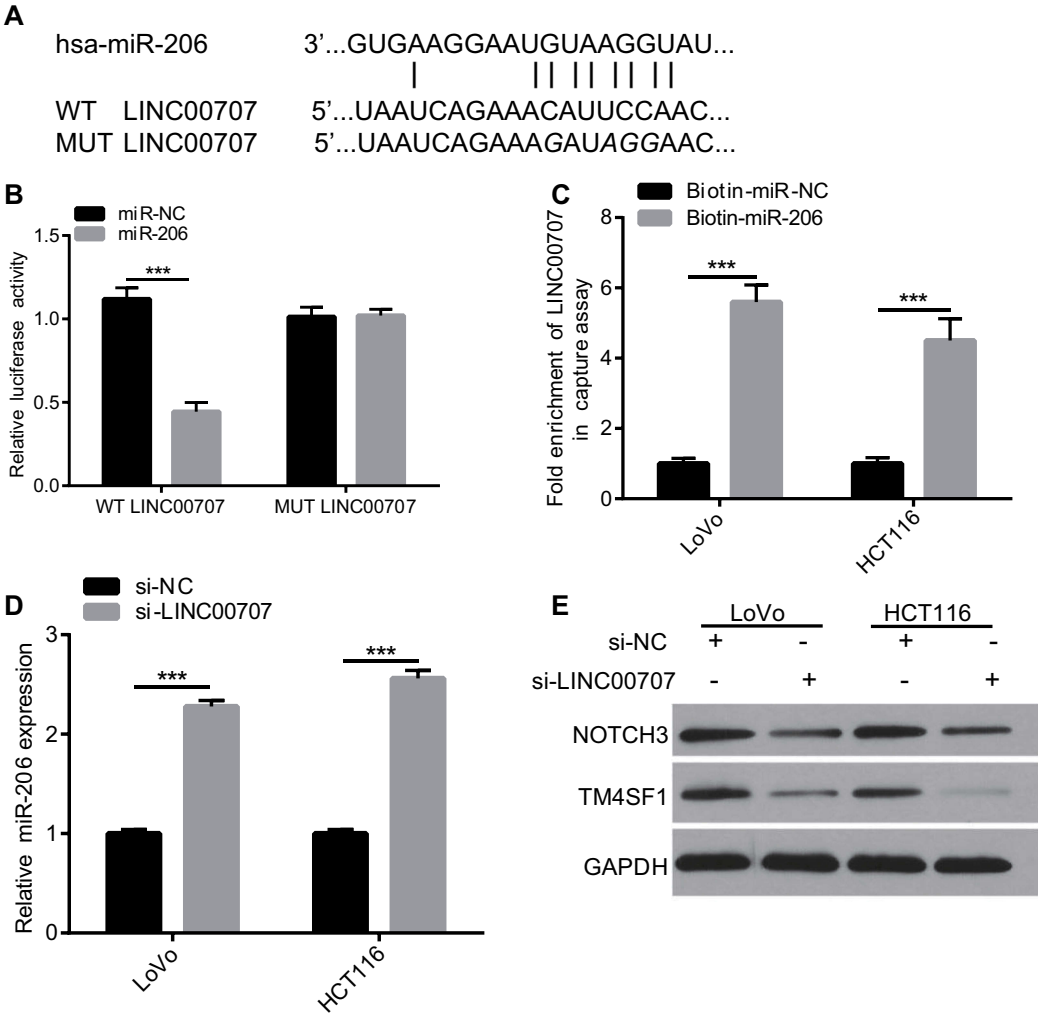

Figure 4 LINC00707 acts as a molecular sponge for miR-206 and indirectly modulates the expression of its targets NOTCH3 and TM4SF1. (**A**) The predicted binding sites of miR-206 on the sequence of LINC00707. The binding sites were highlighted by capital letter. (**B**) Luciferase reporter assay was performed to detect luciferase activity after cotransfection of cells with WT-LINC00707 or MUT-LINC00707 and miR-206 mimic. (**C**) Biotin-coupled miRNA capture assay was performed to measure the expression of LINC00707 in LoVo and HCT116 cells transfected with biotin-miR-206 or biotin-miR-NC. (**D**) The expression of miR-206 in LoVo and HCT116 cells transfected with si-LINC00707 or si-NC was measured with quantitative reverse transcription PCR. (**E**) The protein expression of NOTCH3 and TM4SF1 in LoVo and HCT116 cells transfected with si-LINC00707 or si-NC was measured with Western blotting. ***$P<0.001$.
Abbreviations: MUT, mutant; NC, negative control; WT, wild-type.

regulation of miR-206 expression by LINC00707. Therefore, the effects of LINC00707 on CRC cell proliferation, migration, and invasion may be explained, at least in part, by its role as a molecular sponge of miR-206.

miR-206 inhibits cell proliferation and migration through the downregulation of NOTCH3 expression.[15] The expression of NOTCH3, identified as the third mammalian notch receptor, was significantly upregulated in CRC, and NOTCH3 silencing suppressed the proliferation and tumorigenic properties of CRC cells.[25] Park et al reported that miR-206 suppresses PGE2-induced cell proliferation, migration, and invasion by targeting TM4SF1.[16] TM4SF1 is a member of the tetraspanin L6 domain family and its expression was shown to be upregulated in primary CRC. In addition, TM4SF1 regulates the metastatic potential of CRC cells through the activation of epithelial–mesenchymal transition regulator.[26] These reports have indicated that NOTCH3 and TM4SF1 are targets of miR-206 and act as important regulators in the development and metastases of CRC. In our study, we found that LINC00707 knockdown significantly decreased the protein expression of NOTCH3 and TM4SF1 in both LoVo and HCT116 cells. These results further indicated that LINC00707 promotes CRC cell proliferation and metastasis by sponging miR-206.

The present study has some limitations. Follow-up study was not carried out in present study. The potential prognostic role of LINC00707 should be evaluated in the further study. Although we identified miR-206 as a target of LINC00707,

LINC00707 may have other potential targets. Further research is warranted to study how LINC00707 differentiates and regulates these microRNAs. Moreover, miR-206 has many target genes, including the target genes we detected. Furthermore, we did not verify the expression profile of LINC00707 in CRC cell lines and the effect of LINC00707 knockdown on tumor inhibition in animals. Despite these drawbacks, our study highlights the potential role of LINC00707 in CRC development and metastasis.

In conclusion, we demonstrate the high expression of LINC00707 in CRC tissues. Knockdown of LINC00707 expression significantly inhibits CRC cell growth and metastasis by sponging miR-206. These findings suggest that LINC00707 may serve as a potential target for CRC treatment.

Acknowledgments

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Disclosure

The authors declare that they have no competing interests.

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Supplementary material

Table S1 The clinical information of the 40 patients analyzed in the study

Patient no.	Age (years)	Gender	Tumor size	Lymphatic metastasis	Distant metastasis
1	45	Female	<5 cm	No	No
2	65	Male	<5 cm	No	No
3	38	Male	<5 cm	No	No
4	59	Female	<5 cm	No	No
5	37	Female	<5 cm	No	No
6	46	Male	<5 cm	No	No
7	44	Female	≥5 cm	No	No
8	40	Male	<5 cm	No	No
9	56	Male	≥5 cm	Yes	Yes
10	31	Female	≥5 cm	No	Yes
11	69	Male	≥5 cm	Yes	Yes
12	59	Male	<5 cm	No	No
13	43	Male	≥5 cm	Yes	No
14	40	Female	<5 cm	No	Yes
15	76	Male	≥5 cm	Yes	Yes
16	32	Male	<5 cm	No	No
17	45	Male	<5 cm	Yes	No
18	65	Female	<5 cm	No	No
19	61	Male	<5 cm	Yes	No
20	68	Male	≥5 cm	Yes	Yes
21	60	Male	≥5 cm	Yes	Yes
22	64	Female	<5 cm	No	Yes
23	68	Male	<5 cm	Yes	No
24	63	Male	<5 cm	Yes	Yes
25	38	Female	<5 cm	No	No
26	65	Male	<5 cm	No	No
27	61	Male	≥5 cm	Yes	Yes
28	36	Male	≥5 cm	Yes	No
29	72	Female	<5 cm	No	No
30	70	Male	≥5 cm	Yes	Yes
31	62	Female	≥5 cm	Yes	Yes
32	69	Female	<5 cm	No	Yes
33	67	Male	<5 cm	Yes	No
34	60	Female	<5 cm	No	No
35	54	Female	<5 cm	No	No
36	64	Male	≥5 cm	Yes	Yes
37	63	Male	≥5 cm	Yes	Yes
38	68	Female	≥5 cm	Yes	Yes
39	60	Male	≥5 cm	Yes	Yes
40	70	Male	<5 cm	No	No

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