

Knockdown of LINC01224 Suppresses Colon Cancer Progression by Sponging miR-485-5p to Downregulate MCL1

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Background: Colon cancer (CC) is the most commonly occurring malignant tumor in the world. The current cancer treatment options have been less effective especially in the advanced stages of CC and patients have poor overall survival. Hence, there is an urgent need to explore novel molecular therapeutic targets for CC treatment.

Methods: qRT-PCR was performed to detect the levels of lncRNA LINC01224 (LINC01224), microRNA-485-5p (miR-485-5p), MCL1 in CC tumor tissues or cell lines. Two si-RNAs against LINC01224 were used to silence the level of LINC01224, and CCK-8 assay, colony formation assay, and transwell assay were performed to explore the role of LINC01224 on the proliferation, migration, and invasion of CC cell lines. Kaplan–Meier method was applied for evaluating the association between LINC01224 level and the overall survival of CC patients. Through bioinformatics analysis, we found that LINC01224 sponged miR-485-5p and consequently targeted MCL1. Dual-luciferase reporter assay, RNA pull-down assay, qRT-PCR, and Western blot assay were conducted for verification of the interactions among LINC01224, miR-485-5p, and MCL1. Furthermore, the role of LINC01224/miR-485-5p/MCL1 axis in CC progression was investigated by CCK-8 assay, colony formation assay, and transwell assay.

Results: LINC01224 was highly expressed in CC tumor tissues and CC cell lines, and its expression was associated with the overall survival of CC patients. The LINC01224-siRNAs (si-LINC01224) markedly suppressed the level of LINC01224 in CC cell lines (HT29 and SW480 cells) and consequently significantly suppressed the proliferation, migration, and invasion of the HT29 and SW480 cells. LINC01224 was verified to sponge miR-485-5p and consequently targeted MCL1. MiR-485-5p inhibitor or MCL1 overexpression (MCL1 OE) markedly restored the repressive effect of the si-LINC01224 pool on MCL1 expression level, as well as proliferation, migration, and invasion of HT29 and SW480 cells.

Conclusion: This study identified LINC01224/miR-485-5p/MCL1 axis as a novel molecular therapeutic target involved in CC progression.

Keywords: colon cancer, LINC01224, miR-485-5p, MCL1

Introduction

Colon cancer (CC) is the most commonly occurring malignant tumor in the world and leads to a major global health problem.¹ In China, its incidence is rapidly rising.² Until now, several therapies: operation, radiotherapy, and chemotherapy were major solid tumor treatments of CC. Moreover, multi-modality therapy has been previously used in the treatment of CC patients, which made the incidence and mortality rate decreased in recent years.³ However, most of the diagnosed CC cases

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are always at an advanced stage, leading to a poor prognosis.⁴ Several common chemotherapeutic agents used for CC treatment have been confirmed with limited effectiveness, toxicity, and drug resistance.⁵ Therefore, more effective therapies are urgently needed. Accumulating evidence has verified that several biomarkers as molecular targets participate in cancer progression. In this study, we aim to determine potential diagnostic biomarkers and molecular therapeutic targets.

Long noncoding RNAs (lncRNAs) are a class of transcribed RNA molecules longer than 200 nucleotides.⁶ In recent years, the roles of many lncRNAs have been extensively studied in cancer progression and carcinogenesis.⁷ More and more attention has been focused on their effects on tumors. Multiple lncRNAs have been reported to be abnormally expressed in cancer tumors, and have been regarded as oncogenes or tumor suppressors.^{8,9} In the process of exploring the molecular therapeutic targets of CC, several lncRNAs such as lncRNA LINC00460,¹⁰ lncRNA NEAT1,¹¹ and lncRNA STEAP3-AS1¹² have been verified as oncogenes. Notably, mostly lncRNAs act as a sponge for microRNAs (miRNAs) to regulate the expression of the target gene in CC treatment. Previous studies have confirmed that lncRNAs can serve as competing endogenous RNAs (ceRNAs) or sponges for miRNAs, further acting on the target mRNAs.¹³ Hence, exploring the lncRNA-miRNA axis is essential for the development of molecular therapeutic targets.

The lncRNA LINC01224 reportedly promotes Epithelial Ovarian Cancer progression by sponging miR-485-5p and consequently increasing PAK4 expression.¹⁴ Whereas the role of LINC01224 in CC has not been investigated till now. In this study, for the first time, we systematically explored the role of LINC01224 in CC progression and further investigated the underlying mechanism.

Materials and Methods

Samples

The tumoral tissues and adjacent normal tissues from 52 patients with CC were obtained from the institutional review board of Taizhou Hospital of Zhejiang Province. The informed consent of the patients with CC was provided according to the declaration of Helsinki. And this study was approved by the Ethics Committee of Taizhou Hospital of Zhejiang Province, the approval number is

Table 1 The Clinical Parameters of the Studied CC Patients

Variable	LINC01224 Expression		P value
	Low(n=26)	High(n=26)	
Age			
<60	12	10	0.575
≥60	14	16	
Gender			
Male	11	13	0.578
Female	15	13	
Tumor size			
<5 cm	21	3	0.000
≥5 cm	5	23	
Histological grade			
Grade I+II	20	9	0.002
Grade III+IV	6	17	
Lymph node metastasis			
Yes	8	22	0.000
No	18	4	
Distant metastasis			
M0	16	18	0.560
M1	10	8	

[KY-E-2019-10-20]. The clinical parameters of the patients were shown in Table 1.

Cells and Plasmids

The CC cell lines (HCT116, HT-29, SW620, and SW480) and normal human colonic mucosa cells (NCM460) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The si-RNAs (si-LINC01224#1, si-LINC01224#2, si-NC) were designed and synthesized by TransGen Biotech Co., Ltd. (Beijing, China). The miR-485-5p mimics/inhibitor was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Cell transfection was achieved by using the Lipofectamine 2000 according to the manufacturer's information (Invitrogen, Carlsbad, CA, USA).

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted by Trizol reagent according to the manufacturer's information (Invitrogen, Carlsbad, CA, USA). cDNA synthesis was achieved by using PrimeScriptTMRT Reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). The qRT-PCR analysis was performed by using the SYBR Green Premix ExTaq PCR kit

(Beijing Cowin Biotech., China) on the ABI7300 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The primers used in this study were designed by Primer 5.0.

Cell Counting Kit- 8 (CCK- 8) Assay

CCK-8 assays were performed to explore the effect of LINC01224 knockdown on HT-29 and SW480 cell proliferation abilities. In brief, cells in different groups were plated on a 96-well plate. CCK-8 reagent (Keygen, Jiangsu, China) was added at 0 h, 24 h, 48 h, and 72 h after transfection. Afterward, the absorbance at 450 nm was detected by a microplate reader.

Colony Formation Assays

Colony formation assay was performed to investigate the effect of LINC01224 knockdown on CC cell clonogenic activities. After transfection for 48 h, the cells (5×10^3 cells per well) were seeded on a 6-well plate. Following culture for two weeks, the cells were fixed with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) and stained with 0.1% crystal violet dye (Sigma-Aldrich, St. Louis, MO, USA). Colony number was counted using an inverted microscope.

Transwell Assay

The migration or invasion abilities of CC cells were determined using transwell chambers (Corning Company, NY, USA). Briefly, cells were resuspended and planted into upper chambers (about 1×10^4 cells) with or without the pre-coated Matrigel (BD Bioscience, Waltham, MA, USA). Meanwhile, an 800 μ L medium with 30% FBS was planted into the lower chamber. The chamber was incubated for 24 h at 37 °C. Subsequently, the transmigrated cells were fixed with stained with crystal violet (Sigma-Aldrich, St. Louis, MO, USA). The migrated or invaded cells were photographed and counted by microscopy (Nikon, Tokyo, Japan).

Animal Experiments and Xenograft Collection

The nude mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). A suspension of HT29 cells (about 5×10^6 cells) was injected subcutaneously into the nude mice. The tumor volume was greater than 100 mm³, and the mice were selected for further experiments. Following the selection,

intratumoral multipoint injection with si-NC or si-LINC01224#1 (50 nM) was performed. Each group contained six mice. After treatment, the mice were separately sacrificed at 7 days, 14 days, 21 days, and 28 days, then the tumors were excised for evaluation. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) and the welfare of the laboratory animals strictly follows “The Guidance of The Good Treatment of Laboratory Animals” issued by the National Defense Science and Technology, as well as Five Freedom-Principles. The animal experiment authorized by the Animal Ethics Committee of Taizhou Hospital of Zhejiang Province.

Western Blot

Cells were lysed with RIPA buffer and quantified with a BCA Protein Assay Kit (Beyotime, Beijing, China). Total protein (40 μ g) were loaded to SDS-PAGE gel and transferred onto a PVDF membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% skim milk and incubated with MCL1 antibody (1: 1000; Beyotime, Beijing, China)(catalog:AF1390). After incubation, the secondary antibody HRP-labeled Goat Anti-Rabbit IgG (H+L)) (1: 1000; Beyotime, Beijing, China) (catalog:A0208) was used to incubate the membrane. The membrane was consequently visualized with an ECL reagent (Beyotime, Beijing, China). GAPDH was used as an internal control.

Dual-Luciferase Reporter Assay

Through starbase 2.0, we found the miR-485-5p binding site in LINC01224. The LINC01224 wide type (LINC01224 WT) and mutant type (LINC01224 Mut) reporter vectors were established by Beijing TransGen Biotech Co., (Beijing, China). The reporter plasmids were co-transfected with either miR-485-5p mimics or a negative control into H293T cells. After transfection for 48 h, the cells were lysed and the relative luciferase activities were detected using a dual-luciferase reporter assay system (Promega, Madison, WI, USA).

RNA Pull-Down Assay

For RNA pull-down assay, the streptavidin-coated magnetic beads (Life Technologies, CA, USA) were covered by biotinylated LINC01224 (Bio-LINC01224) and Bio-NC according to its instruction and transfected into 1×10^6 HT29 cells at a final concentration of 50 nM for 48 h before harvest. Subsequently, 0.7 mL lysis buffer (5 mM MgCl₂, 100 mM

KCl, 20 mM Tris (pH 7.5), 0.3% NP-40) and complete protease inhibitor cocktail (Roche Applied Science, IN) were added into the cell pellets, then the cell lysates were incubated together with the RNA-tagged beads for the co-immunoprecipitation (Invitrogen, Carlsbad, CA, USA). The RNA-RNA complexes were subsequently collected by centrifugation at 10,000r for 10 min and then the miR-485-5p enrichment level was detected with qRT-PCR analysis.

Statistical Analysis

The statistical analysis was performed using SPSS 20.0 (SPSS, Chicago, IL, USA). The difference between two groups was calculated by Student's *t*-test. The significance among multiple groups was calculated by ANOVA with Bonferroni correction. The survival curves were assessed by Kaplan–Meier analysis. $p < 0.05$ was considered statistically significant.

Results

Expression Level of LINC01224 in CC Tumor Tissues and Several Cell Lines

The qRT-PCR assay revealed that the expression level of LINC01224 was higher in several CC cell lines (HCT116, HT-29, SW620, and SW480) and CC tumor tissues, as compared with levels observed in NCM460 cells and normal tissues (Figure 1A and B). The patients were divided into low expression level group ($n=26$) and high expression level group ($n=26$). The KM survival curve indicated that patients with higher LINC01224 levels have a lower survival percent (Figure 1C). The clinicopathological data was assessed by chi-square test and summarized in Table 1. The analysis demonstrated that LINC01224 level is associated with stage, lymph node metastasis, and tumor size, not with age and sex.

Knockdown of LINC01224 Suppressed Proliferation, Migration, and Invasion of CC Cells

As shown in Figure 2A, two si-RNAs against LINC01224 effectively repressed the LINC01224 level (Figure 2A). CCK-8 assay illustrated that LINC01224 knockdown significantly suppressed the OD 450 values at 72 h in HT29 and SW480 cells (Figure 2B). Colony formation assay showed that silencing LINC01224 significantly decreased the colony numbers as compared with si-NC groups (Figure 2C). Transwell assays demonstrated that LINC01224 knockdown significantly suppressed migration and invasion abilities of HT29 and SW480 cells (Figure 2D and E).

Xenografts volume and weight were measured for evaluation. Measurements of the tumor xenografts revealed that LINC01224 knockdown markedly reduced tumor volume and weight (Figure 2F and G).

LINC01224 Acts as a Sponge for miR-485-5p

Through the Starbase online database, we found a binding site of miR-485-5p in LINC01224 sequence (Figure 3A). For evaluation of the interaction between miR-485-5p and LINC01224, a series of functional trials were performed. Dual-luciferase reporter assay showed that miR-485-5p overexpression markedly suppressed the luciferase activity of H293T cells in LINC01224 WT group, but not work in LINC01224 Mut group (Figure 3B). RNA pull-down assay found a significant enrichment of miR-485-5p with bio-LINC01224, compared with input control (Figure 3C). qRT-PCR revealed that miR-485-5p levels were markedly up-regulated in si-LINC01224 pool group cells, compared with levels observed in si-NC group (Figure 3D). The

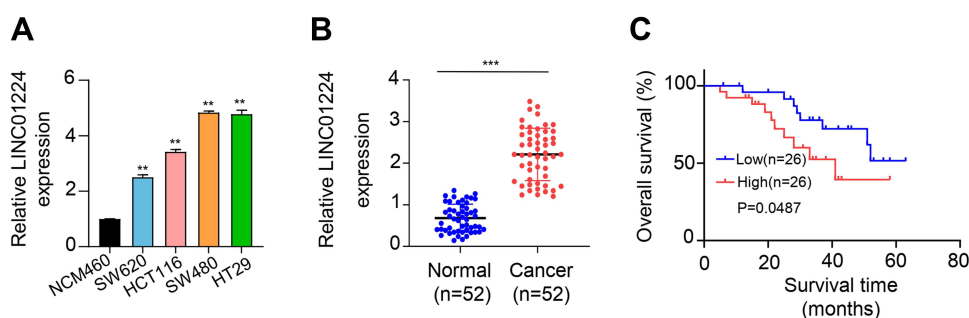


Figure 1 Expression level of LINC01224 in CC tumor tissues and several cell lines. (A) Expression levels of LINC01224 in several colon cancer cell lines (HCT116, HT-29, SW620 and SW480) and control NCM460 cells were detected by qRT-PCR. (B) Expression levels of LINC01224 in colon cancer tumor tissues and adjacent normal tissues were detected by qRT-PCR. (C) Kaplan–Meier survival curves for patients with colon cancer were plotted according to high or low LINC01224 expression level. $**p < 0.01$. $***p < 0.001$.

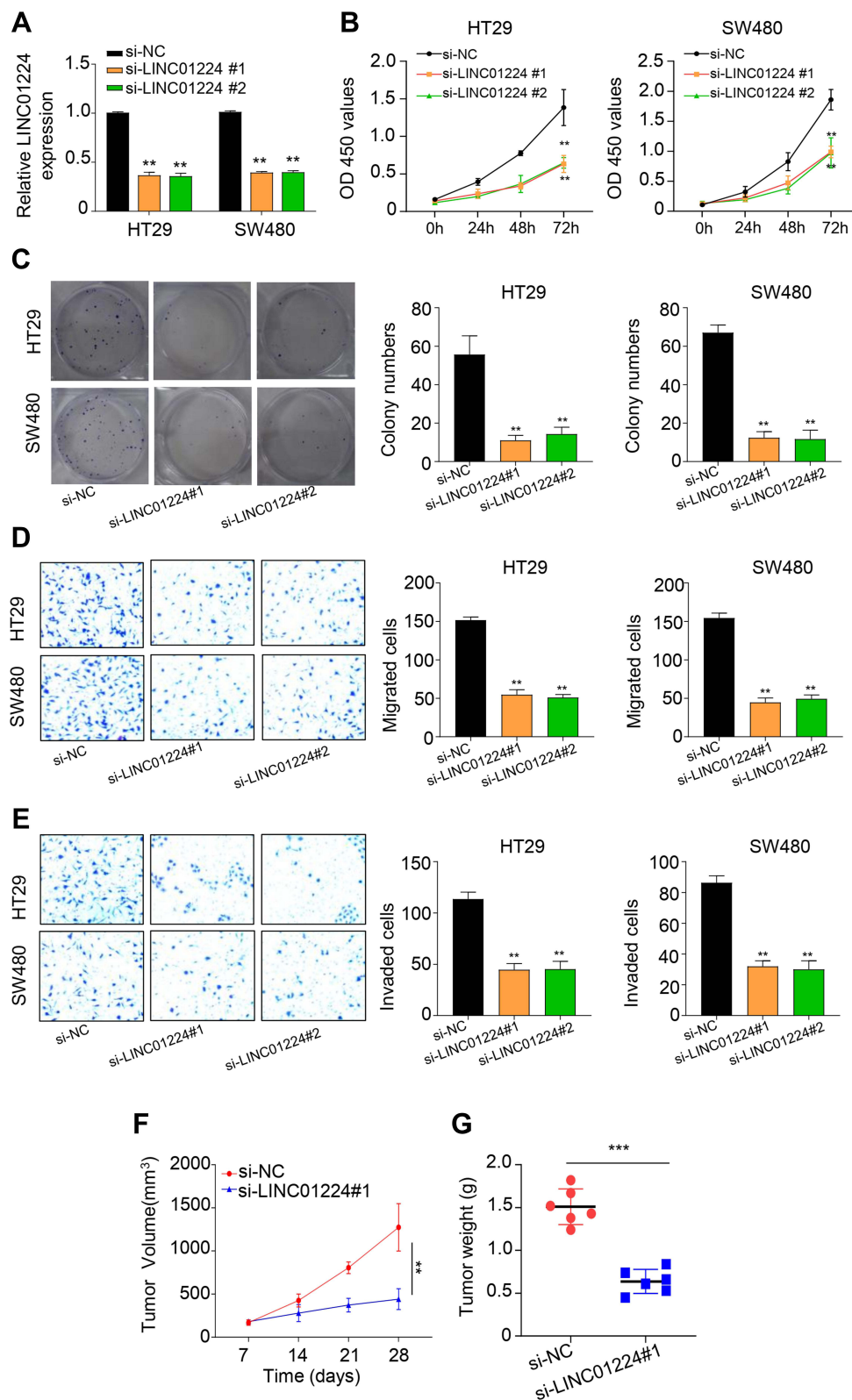


Figure 2 Knockdown of LINC01224 suppressed proliferation, migration and invasion of CC cells. **(A)** qRT-PCR revealed that si-RNA LINC01224#1 or si-RNA LINC01224#2 effectively suppressed the level of LINC01224 in HT29 and SW480 cells. **(B)** CCK-8 assay illustrated that LINC01224 knockdown significantly suppressed the OD 450 values at 72 h in HT29 and SW480 cells. **(C)** Colony formation assay showed that LINC01224 knockdown significantly decreased the colony numbers as compared with si-NC group. **(D)** Transwell assays demonstrated that LINC01224 knockdown significantly suppressed migration ability of HT29 and SW480 cells. **(E)** Transwell assays demonstrated that LINC01224 knockdown significantly suppressed invasion ability of HT29 and SW480 cells. **(F and G)** LINC01224 knockdown markedly suppressed tumor volume and weight. ** $p < 0.01$. *** $p < 0.001$.

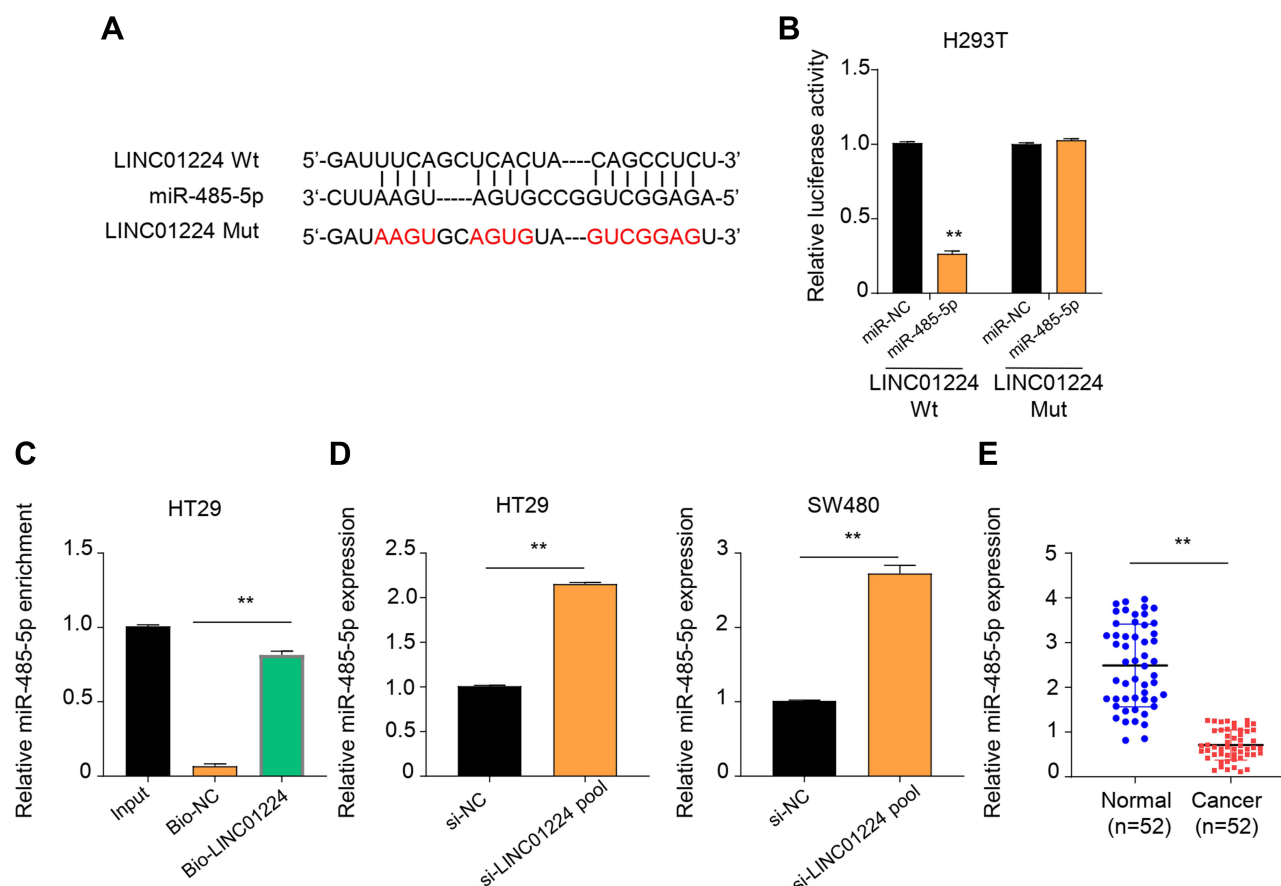


Figure 3 LINC01224 acts as a sponge for miR-485-5p. (A) Starbase online database predicted a binding site of miR-485-5p in LINC01224 sequence. (B) Dual-luciferase reporter assay was used to evaluate the interaction between miR-485-5p and LINC01224. The results showed that miR-485-5p overexpression markedly suppressed the luciferase activity of H293T cells in LINC01224 wildtype (WT) group, but not work in LINC01224 mutant (Mut) group. (C) The binding of LINC01224 to miR-485-5p detected by RNA pull-down. RNA pull-down assay found a significant enrichment of miR-485-5p with bio-LINC01224, compared with input control. (D) qRT-PCR revealed that miR-485-5p levels were markedly up-regulated in si-LINC01224 pool group cells, compared with levels observed in si-NC pool group. (E) qRT-PCR detected that the expression level of miR-485-5p in colon cancer tumor tissues and adjacent normal tissues. The expression level of miR-485-5p was lower in CC tumor tissues than that in normal tissues. $^{**}p < 0.01$.

expression level of miR-485-5p was lower in CC tumor tissues than that in normal tissues (Figure 3E).

Interaction Between MCL1 and miR-458-5p

Starbase online database predicted that a binding site of miR-458-5p in MCL1 sequence (Figure 4A). Dual-luciferase reporter assay results showed that MCL1 WT reporter was strongly inhibited by miR-485-5p, whereas MCL1 Mut reporter was not affected by miR-485-5p (Figure 4B). In miR-485-5p mimics groups, the RNA and the protein levels of MCL1 were markedly inhibited in HT29 and SW480 cells (Figure 4C and D). The level of MCL1 was obviously higher in CC tumor tissues than that in normal tissues (Figure 4E).

LINC01224 Inhibited MCL1 Expression by Sponging miR-455-5p

In HT29 and SW480 cell lines, si-LINC01224 pool markedly suppressed the mRNA and protein level of MCL1, whereas

miR-485-5p inhibitor (miR-485-5p inh) or MCL1 overexpression (MCL1 oe) restored these effects of si-LINC01224 pool (Figure 5A and B). Furthermore, the functional experiments displayed that miR-485-5p inh or MCL1 oe markedly restored the repressive effect of si-LINC01224 pool on the cell proliferation (Figure 5C and D), cell migration (Figure 5E) and invasion (Figure 5F). These data suggest that LINC01224 may be a potential competing endogenous RNA to miR-485-5p. And silence of LINC01224 can aggravate the miR-485-5p-induced MCL1 downregulation.

Discussion

This study was primarily conducted to identify possible molecular therapeutic target for CC treatment. LncRNAs have been shown to be involved in the regulation of various cellular processes. Studies have shown that lncRNA can effectively regulate the epithelial-mesenchymal transition (EMT) by sponging miR-433-3p.¹⁰ It has also been shown

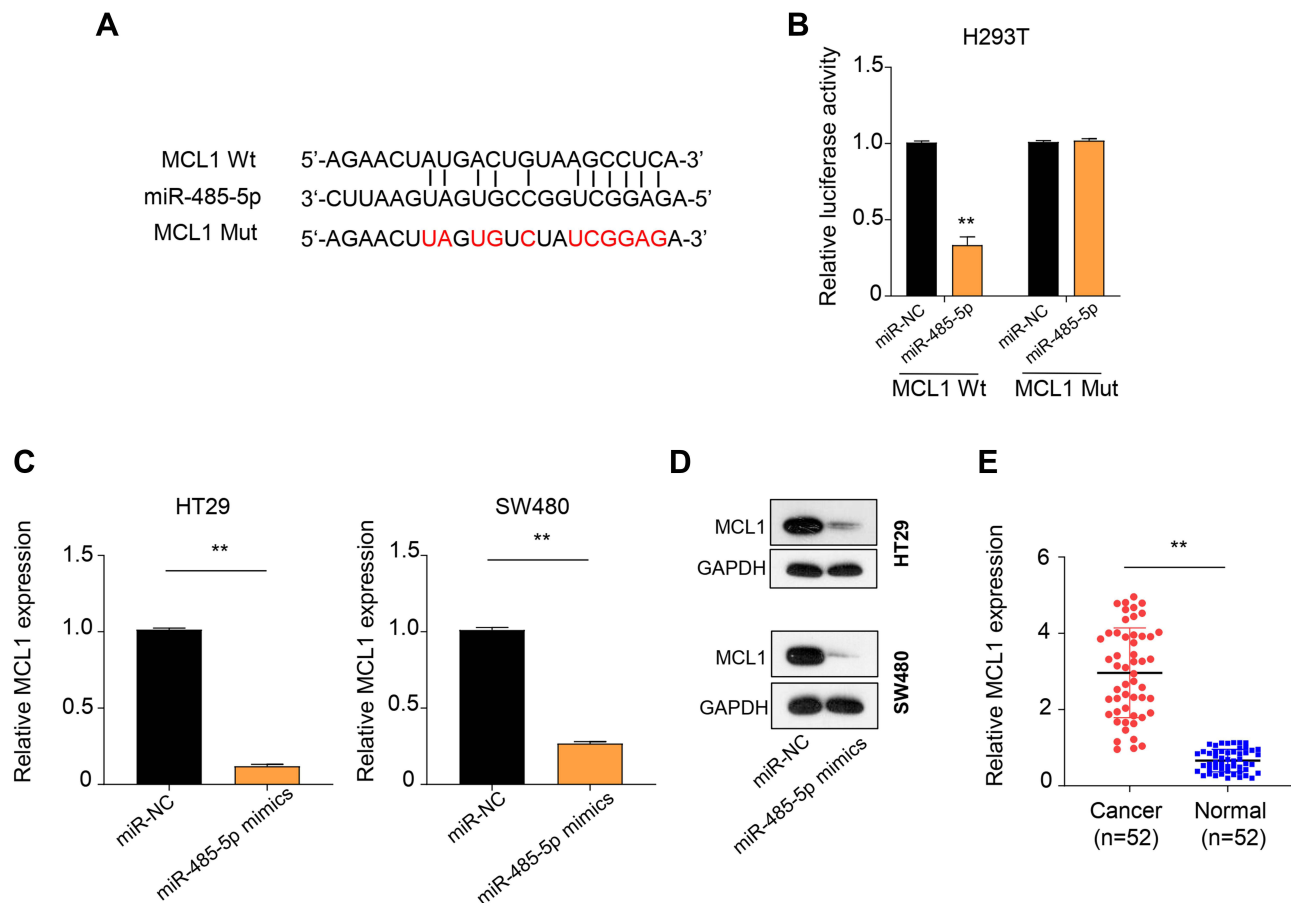


Figure 4 Interaction between MCL1 and miR-485-5p. **(A)** Starbase online database predicted that a binding site of miR-485-5p in MCL1 sequence. **(B)** Dual-luciferase reporter assay was used to evaluate the interaction between miR-485-5p and MCL1. The results showed that MCL1 WT reporter was strongly inhibited by miR-485-5p, whereas MCL1 Mut reporter was not affected by miR-485-5p. **(C and D)** The mRNA and the protein levels of MCL1 in HT29 and SW480 cells were markedly inhibited in miR-485-5p mimics groups. **(E)** qRT-PCR results revealed that the level of MCL1 was obviously higher in CC tumor tissues than that in normal tissues. ** $p < 0.01$.

to regulate the proliferation, migration and arrest of colon cancer cells at the G0–G1 phase,¹² and also regulate the invasion and migration of CC cells.¹¹

In this study, we first investigated the role of LINC01224 in CC progression. We found a high level of LINC01224 in CC tumors and cells, which consequently resulted in a poor prognosis. We confirmed that LINC01224 level was related with tumor size, histological grade and lymph node metastasis. These results indicated that LINC01224 can be regarded as a biomarker for CC. Furthermore, we found that an in vitro knockdown of LINC01224 markedly suppressed the proliferation, migration and invasion of HT29 and SW480 cells. Our in vivo study also showed that the expression level of LINC01224 positively correlated with the tumor volume and weight, indicating that the LINC01224 is an oncogenic lncRNA involved in CC progression and repressing its expression could significantly inhibit the progression of colon cancer.

Mostly, lncRNAs serve as ceRNAs to sponge miRNAs, consequently regulating the target mRNA. In this study, we confirmed that LINC01224 acted as a sponge for miR-485-5p and further regulated the expression level of its target mRNA myeloid cell leukemia sequence 1 (MCL1). Accumulating studies have revealed the critical roles of many miRNAs in cancer development, as they serve as oncogenes or cancer suppressor genes. Recently, miR-485-5p has been studied in multiple tumors, including hepatocellular carcinoma,^{15,16} ovarian cancer,¹⁴ papillary thyroid cancer,¹⁷ breast cancer,¹⁸ colorectal cancer^{19,20} and so on. From previous studies, we found that the levels of miR-485-5p were down-regulated in multiple cancer tumors and cells, and overexpression of miR-485-5p could suppress tumor cell metastasis, migration, invasion and proliferation, indicating that miR-485-5p could function as a tumor suppressor in multiple cancers including colorectal cancer. In this study, the results confirmed that miR-485-5p level was markedly lower in CC tumor tissues than adjacent normal

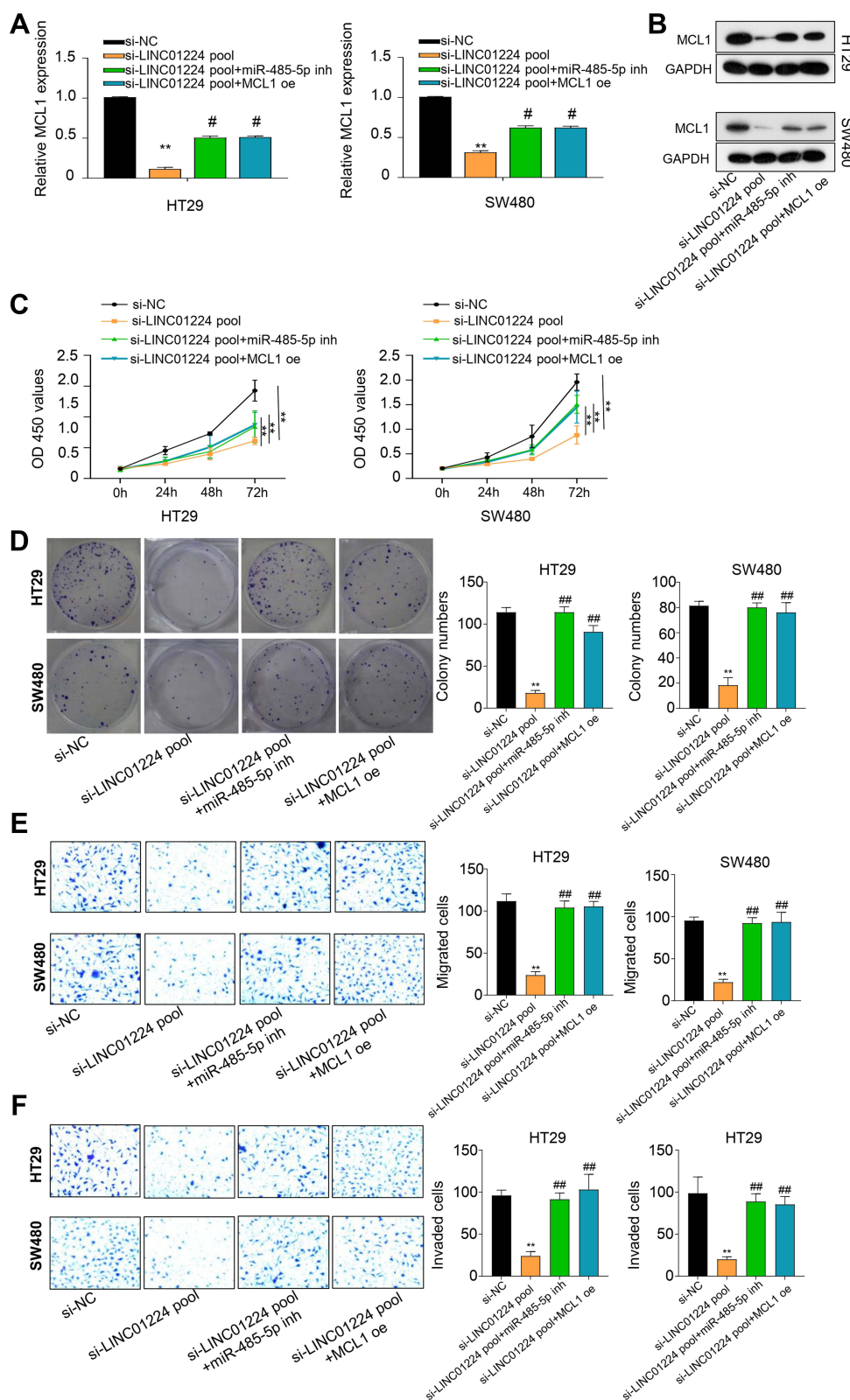


Figure 5 LINC01224 inhibited MCL1 expression by sponging miR-455-5p. (A and B) In HT29 and SW480 cell lines, si-LINC01224 pool markedly suppressed the mRNA and protein level of MCL1, whereas miR-485-5p inhibitor (miR-485-5p inh) or MCL1 overexpression (MCL1 oe) restored the effects of si-LINC01224 pool. (C–F) CCK-8 assay, colony formation assay, transwell assays with Matrigel displayed that miR-485-5p inh or MCL1 oe markedly restored the repressive effect of si-LINC01224 pool on the cell proliferation (C), cell lone formation (D), cell migration (E) and invasion (F). ** $p < 0.01$ versus si-NC group. # $p < 0.01$, ## $p < 0.001$ versus si-LINC01224 pool group.

tissues, which is consistent with previous researches. MCL1, the miR-485-5p target mRNA observed in this study, role in tumorigenesis was also studied in previous research.²¹ MCL1 was one important anti-apoptotic member of the BCL-2 family.²² Its involvement in multiple myeloma,^{23,24} osteosarcoma,²⁵ and colon cancer^{21,26,27} has been reported. The overexpression of miR-125a-5p, the tumor suppressor, significantly suppressed the expression level of MCL1, and restoration of MCL1 in CC cells reversed the cell proliferation inhibition and apoptosis stimulation caused by miR-125-5p.²¹ Lu et al reported that MCL1 si-RNA could be delivered by DOTAP and MPEG-PCL hybrid micelles for the treatment of CC in vivo and in vitro, which was proved to induce the apoptosis of C26 CC cells and suppress the growth of C26 cells with high safety.²⁶ Hence, we concluded that MCL1 is a target gene involved in CC progression.

In summary, in this study, we identified the LINC01224/miR-485-5p/MCL1 axis involvement in CC progression and demonstrated that LINC01224 knock-down could increase the level of miR-485-5p (the tumor suppressor), and consequently inhibit the level of MCL1 (an oncogene for CC progression). Overall, these results highlighted LINC01224/miR-485-5p/MCL1 axis as a possible molecular therapeutic target involved in the progression of CC.

Disclosure

The authors report no conflicts of interest in this work.

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