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LINC00346 accelerates the malignant progression of colorectal cancer *via* competitively binding to miRNA-101-5p/MMP9

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Abstract. – OBJECTIVE: To clarify the promotive effect of LINC00346 on the malignant progression of colorectal cancer (CRC) by mediating miRNA-101-5p/MMP9 axis.

PATIENTS AND METHODS: Expression pattern of LINC00346 in 46 paired CRC tissues and adjacent normal tissues was determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Correlation between LINC00346 level and prognosis of CRC patients was analyzed, and the LINC00346 level in CRC cell lines was examined as well. Subsequently, potential influences of LINC00346 on cellular behaviors of CRC cells were evaluated through cell counting kit-8 (CCK-8), colony formation, transwell, and wound healing assays. Finally, Dual-Luciferase reporter gene assay was conducted to verify the binding relationship between LINC00346 and miRNA-101-5p/MMP9.

RESULTS: LINC00346 was upregulated in CRC tissues and cell lines. Compared with CRC patients with low level of LINC00346, those with high level suffered a poorer prognosis, and higher metastatic rates (lymph node metastasis and distant metastasis). Transfection of sh-LINC00346 attenuated proliferative, migratory, and invasive abilities of CRC cells. In addition, LINC00346 was confirmed to bind to miRNA-101-5p, and the latter was binding to MMP9. Moreover, the overexpression of miRNA-101-5p decreased colony number, viability, and numbers of migratory and invasive cells.

CONCLUSIONS: LINC00346 is upregulated in CRC and correlated with metastasis and poor prognosis of CRC. LINC00346 accelerates the malignant progression of CRC via targeting miRNA-101-5p/MMP9.

Key Words:
LINC00346, MiRNA-101-5p, Colorectal cancer, Malignant progression.

Introduction

Colorectal cancer (CRC) is a malignant tumor originating from epithelial tissues of intestinal mucosa under the stimuli of multiple pathogenic factors. Globally, the morbidity and mortality of CRC rank the third and fourth of malignancies, respectively[1-4]. The occurrence and progression of tumors are complex involving multiple factors, genetic mutations, and expression variations[5,6]. Currently, the pathogenesis of CRC is believed to be related to a series of pathological processes, including long-term oncogene activation, tumor suppressor inactivation, inheritance of tumor susceptibility genes, and genetic variants[7,8]. With the in-depth researches on tumor biology, CRC progression could be influenced by non-coding RNAs, DNA methylation, histone modifications, chromatin position, and structural changes[9,10].

Long non-coding RNAs (lncRNAs) are non-coding RNAs with 200-100,000 base pairs in length. LncRNAs have been ignored for a long time since they were previously considered as meaningless transcripts[11,12]. However, with the advance of high-throughput gene sequencing technology, the vital function of lncRNAs has been well concerned[13]. LncRNAs not only exert a regulatory role (sense regulation) by acting on its neighboring genes, but also regulate its distant genes (antisense regulation). Therefore, explorations on lncRNA origins are of great significance[14-16]. LncRNAs may serve as important markers and targets for tumor diagnosis and treatment[16]. LINC00346 is abnormally expressed in a variety of malignant tumors and has a certain influence on tumor development. Besides, it has been considered as an oncogene in many tumors based on their different functions[17,18]. However, the biological role and clinical significance of LINC00346 in CRC have not been comprehensively studied.

LncRNAs are classified according to their regulatory methods and functions. They can induce proteins or miRNAs to isolate from chromatin,

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recruit proteins to transport to DNA, and form a complex with two or more proteins[19]. In recent years, miRNA-101-5p has been widely investigated in malignant tumors[20,21]. In this study, the expression patterns of LINC00346 and miRNA-101-5p/MMP9 in CRC were examined, and the role of LINC00346/miRNA-101-5p/MMP9 in the malignant progression of CRC was revealed.

Patients and Methods

CRC Samples

A total of 46 paired CRC tissues and adjacent normal tissues were surgically resected and pathologically diagnosed. None of the patients underwent preoperative anti-tumor therapy. Patients and their families have been fully informed, and this investigation was approved by the Ethics Committee of The First Hospital of Jilin University.

Cell Culture

CRC cell lines (HT29, HCT-8, and HCT-116) and colorectal mucosal cell line FHC were provided by American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) containing 10% fetal bovine serum (FBS; Life Technologies, Gaithersburg, MD, USA) and maintained in a 37°C, 5% CO_2 incubator. Medium was replaced every 2-3 days. Cell passage was conducted at 90% of confluence.

Transfection

Transfection plasmids were provided by GenePharma (Shanghai, China). Cells seeded in the 6-well plates with 70% of confluence were subjected to transfection using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). At 48 h after transfection, the cells were harvested for subsequent experiments.

Cell Proliferation Assay

Cells were seeded in the 96-well plate with 2×10^3 cells per well. Absorbance (A) at 450 nm was recorded at the appointed time points using the cell counting kit-8 (CCK-8) kit (Dojindo Laboratories, Kumamoto, Japan) for depicting the viability curve.

Colony Formation Assay

Cells were seeded in the 6-well plate with 200 cells per well. Each group had 3 replicate wells. Cells were incubated for 2 weeks. By fixation of 95% ethanol and dye with 1% violet crystal for 20 min, colonies were captured for counting under a microscope.

Transwell Assay

Transfected cells for 48 h were digested and adjusted to 5.0×10^5/mL. 200 μL/well suspension was applied in the upper side of transwell chamber (Millipore, Billerica, MA, USA), and 700 μL of medium containing 10% FBS was applied in the bottom side. After 48 h of incubation, the cells that penetrated to the bottom side were subjected to fixation in methanol for 15 min, crystal violet staining for 20 min, and cell counting using a microscope. Penetrating cells were counted in 5 randomly selected fields per sample.

Wound Healing Assay

Cells were seeded in a 6-well plate with 5.0×10^5/well. Until 90% of confluence, a 1 mL pipette tip was used for creating an artificial wound in the confluent cell monolayer. Percentage of wound closure was calculated at 0 and 24 h, respectively.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and purified by DNase I treatment. Extracted RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The obtained cDNA was subjected to qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). β-actin and U6 were used as internal references. Each sample was performed in triplicate, and relative level calculated by $2^{-\Delta\Delta Ct}$ was analyzed by iQ5 2.0 (Bio-Rad, Hercules, CA, USA).

Western Blot

Total protein was extracted from cells or tissues using radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) and loaded for electrophoresis. After transferring on a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), it was blocked in 5% skim milk for 2 h, and incubated with primary antibodies at 4°C overnight and secondary antibodies for 2 h. Ultimately, bands were exposed by enhanced chemiluminescence (ECL; Pierce, Rockville, MD, USA) and analyzed by Image Software (NIH, Bethesda, MD, USA).

Dual-Luciferase Reporter Gene Assay

Cells were co-transfected with pmirGLO-WT/pmirGLO-MUT/pmirGLO and NC/overexpression plasmid using Lipofectamine 2000. After 24 h, co-transfected cells were harvested for determining luciferase activity using a Dual-Luciferase reporter assay system (Promega, Madison, WI, USA).

Statistical Analysis

GraphPad Prism 5 V5.01 (La Jolla, CA, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Intergroup differences were analyzed by the *t*-test. Differences among multiple groups were analyzed using one-way ANOVA, followed by post-hoc test. Kaplan-Meier was introduced for survival analysis, and Log-rank test was conducted to compare differences between the two curves. $p<0.05$ was considered as statistically significant.

Results

High Expression of LINC00346 in CRC

Expression pattern of LINC00346 in 46 paired CRC tissues and adjacent normal tissues was detected by qRT-PCR. The data showed that LINC00346 was upregulated in CRC tissues (Figure 1A, 1B). Identically, LINC00346 was highly expressed in CRC tissues relative to controls (Figure 1C). It is speculated that LINC00346 may serve as a carcinogenic role in CRC.

LINC00346 Expression Was Correlated With Tumor Stage and Overall Survival in CRC Patients

Based on the follow-up data of enrolled CRC patients, the correlation between LINC00346 level and pathological characteristics of CRC was analyzed. LINC00346 level was proved to be positively correlated with lymph node metastasis

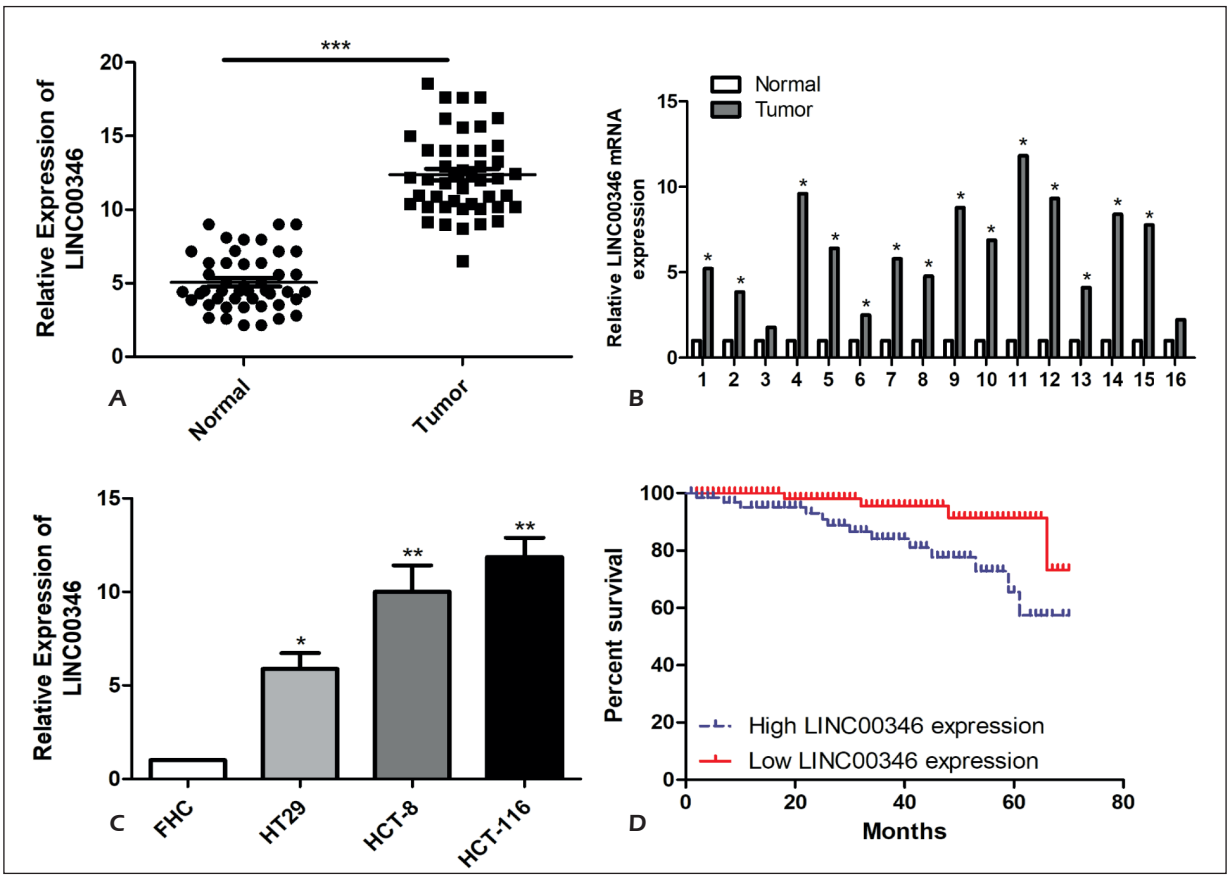

Figure 1. High expression of LINC00346 in CRC. **A**, Relative level of LINC00346 in CRC tissues and adjacent normal tissues. **B**, Relative level of LINC00346 in 16 paired CRC tissues and adjacent normal tissues. **C**, Relative level of LINC00346 in FHC and CRC cell lines. **D**, Kaplan-Meier curves showed overall survival in CRC patients with high or low level of LINC00346.

Table I. Association of LINC00346 expression with clinicopathologic characteristics of colorectal cancer.

Parameters	No. of cases	LINC01308 expression		*p*-value
		Low (%)	High (%)	
Age (years)				0.938
<60	21	12	9	
≥60	25	14	11	
T stage				0.655
T1-T2	27	16	11	
T3-T4	19	10	9	
Lymph node metastasis				0.047
No	26	18	8	
Yes	20	8	12	
Distance metastasis				0.026
No	29	20	9	
Yes	17	6	11	

and distant metastasis, while it did not relate to age and tumor stage of CRC patients (Table I). Survival analysis indicated a poorer prognosis in CRC patients with a high level of LINC00346 (Figure 1D).

Knockdown of LINC00346 Inhibited CRC Cells to Proliferate, Migrate and Invade

Three lines of sh-LINC00346 were constructed, and they all downregulated LINC00346 level in HCT-8 and HCT-116 cells (Figure 2A). In particular, transfection efficacy of sh-LINC00346#1 was the best among the three constructed lentiviruses, and it was selected for the following experiments. CCK-8 assay revealed that transfection of sh-LINC00346#1 markedly reduced the viability in CRC cells (Figure 2B). Besides, the transfection of sh-LINC00346#1 decreased the number of colonies, suggesting the inhibited proliferative ability (Figure 2D). In HCT-8 and HCT-116 cells transfected with sh-LINC00346#1, numbers of migratory and invasive cells were remarkably reduced (Figure 2C). Moreover, the percentage of wound closure decreased in CRC cells with LINC00346 knockdown (Figure 2E). The above data demonstrated that knockdown of LINC00346 attenuated proliferative, migratory, and invasive abilities of CRC cells.

Interaction Between LINC00346 and MiRNA-101-5p in CRC

Through online prediction, miRNA-101-5p was predicted to be the downstream target gene of LINC00346 (data not shown). As qRT-PCR data revealed, miRNA-101-5p was downregulated in CRC tissues and cell lines (Figure 3A, 3B). Transfection of sh-LINC00346#1 upregulated miRNA-101-5p level in HCT-8 and HCT-16 cells (Figure 3C). Conversely, transfection of miRNA-101-5p mimics downregulated LINC00346 level (Figure 3D). Furthermore, a negative correlation between levels of miRNA-101-5p and LINC00346 was identified in CRC tissues (Figure 3E). Relative luciferase activity markedly decreased in CRC cells co-transfected with miRNA-101-5p mimics and pmirGLO-LINC00346-WT (Figure 3F), suggesting that miRNA-101-5p can directly bind to LINC00346.

MiRNA-101-5p Modulated MMP9 Expression in CRC

The biological role of miRNA-101-5p in the malignant progression of CRC was mainly explored. Transfection of miRNA-101-5p mimics decreased colony number, proliferative rate, and migration cell number in CRC cells (Figure 4A, 4B). It was found that both mRNA and protein levels of MMP9 were downregulated after overexpression of miRNA-101-5p in CRC cells (Figure 4C, 4D). A negative correlation was identified between levels of miRNA-101-5p and MMP9 in CRC tissues. To further investigate the relationship between miRNA-101-5p and MMP9, Dual-Luciferase reporter gene assay was carried out. As the data indicated, the luciferase activity markedly decreased in cells co-transfected with pcDNA-MMP9 and pmirGLO-miRNA-101-5p-WT (Figure 4F). The above data indicated that LINC00346 accelerated the malignant progression of CRC *via* miRNA-101-5p/MMP9 axis (Figure 4G).

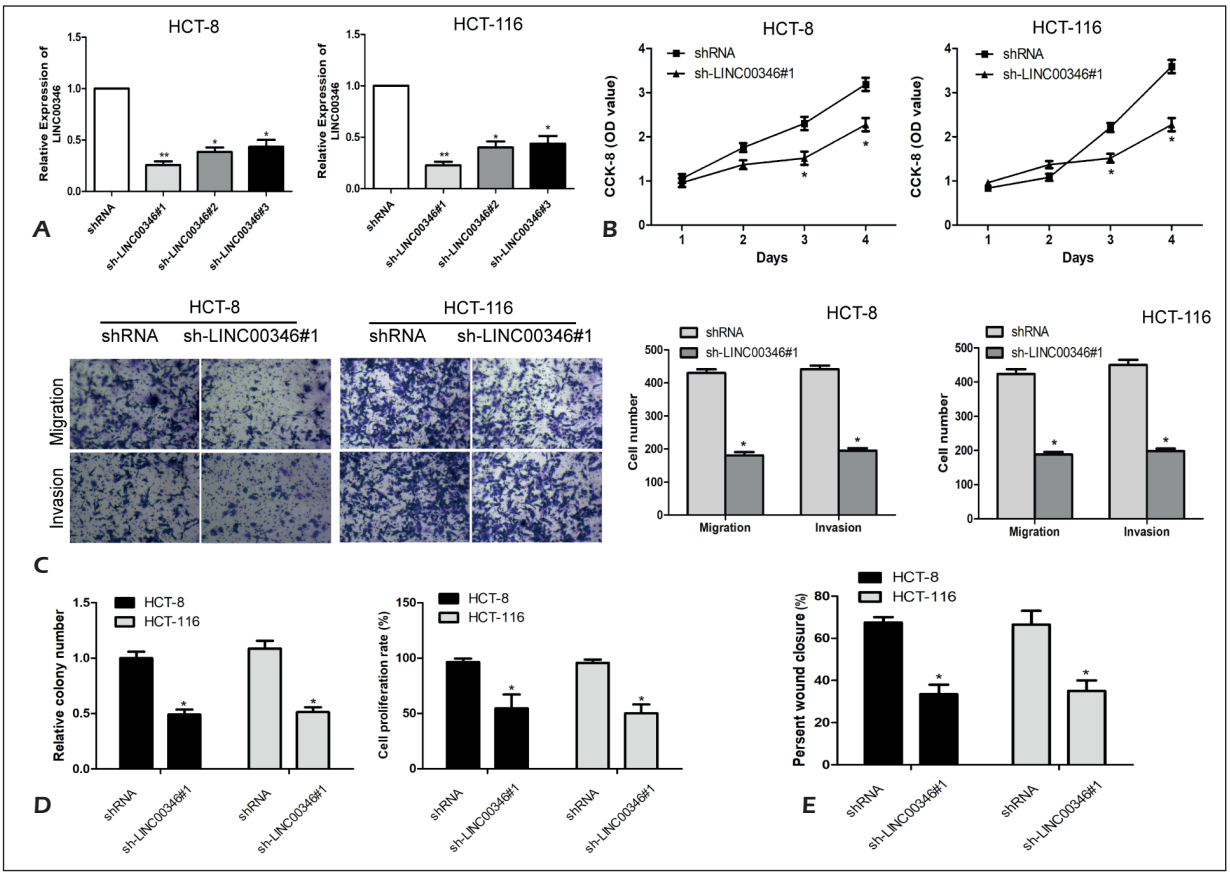

Figure 2. Knockdown of LINC00346 inhibits CRC cells to proliferate, migrate and invade. **A**, Transfection efficacy of sh-LINC00346#1, sh-LINC00346#2 and sh-LINC00346#3 in HCT-8 and HCT-116 cells. **B**, CCK-8 assay shows the viability in HCT-8 and HCT-116 cells transfected with shRNA or sh-LINC00346#1. **C**, Transwell assay shows the migration and invasion in HCT-8 and HCT-116 cells transfected with shRNA or sh-LINC00346#1 (magnification: 40X). **D**, Colony formation assay displays the colony number in HCT-8 and HCT-116 cells transfected with shRNA or sh-LINC00346#1. **E**, Wound healing assay presents the percentage of wound closure in HCT-8 and HCT-116 cells transfected with shRNA or sh-LINC00346#1.

Discussion

Cancer is a complex disease that is accompanied by genetic variation, chromosomal translocation, chromosomal deletion or amplification[9,10]. Non-coding RNAs are transcribed molecules but usually not translated into proteins, and they have been discovered to exert crucial functions in cellular biological functions[10-12]. LncRNA belongs to ncRNAs with diverse functions in biological processes, especially in tumor progression[13-15]. Several lncRNAs have been proved to be abnormally expressed in CRC, including SNHG12, MALAT1, HOTAIR, and H19[15,16]. Their specific regulations in CRC may help to develop novel targets for CRC treatment.

LncRNA binds to homologous RNA or those sharing with similar sequences and folds into complex secondary structures that bind to proteins[11,12]. LncRNA could serve as a competitive endogenous RNA (ceRNA) to competitively bind to a miRNA, thus regulating target gene expressions of this miRNA[19]. The regulatory network lncRNA-miRNA-mRNA is greatly involved in biological processes[16,19]. Previous researches[17,18] revealed the regulatory effect of LINC00346 in tumor behaviors. In this study, LINC00346 was upregulated in CRC tissues, showing a close relationship with the prognosis of CRC patients. It is speculated that overexpression of LINC00346 accelerated the malignant progression of CRC. In addition, LINC00346 stimulated CRC cells to proliferate, migrate, and invade. Relevant factors in tumor microenvironment are variable and have a great impact on the biological functions of cancer cells, including extracellular matrix, body fluid circulation, and endocrine hormones. Therefore, further *in vivo* experiments should be carried out to uncover the role of LINC00346 in CRC.

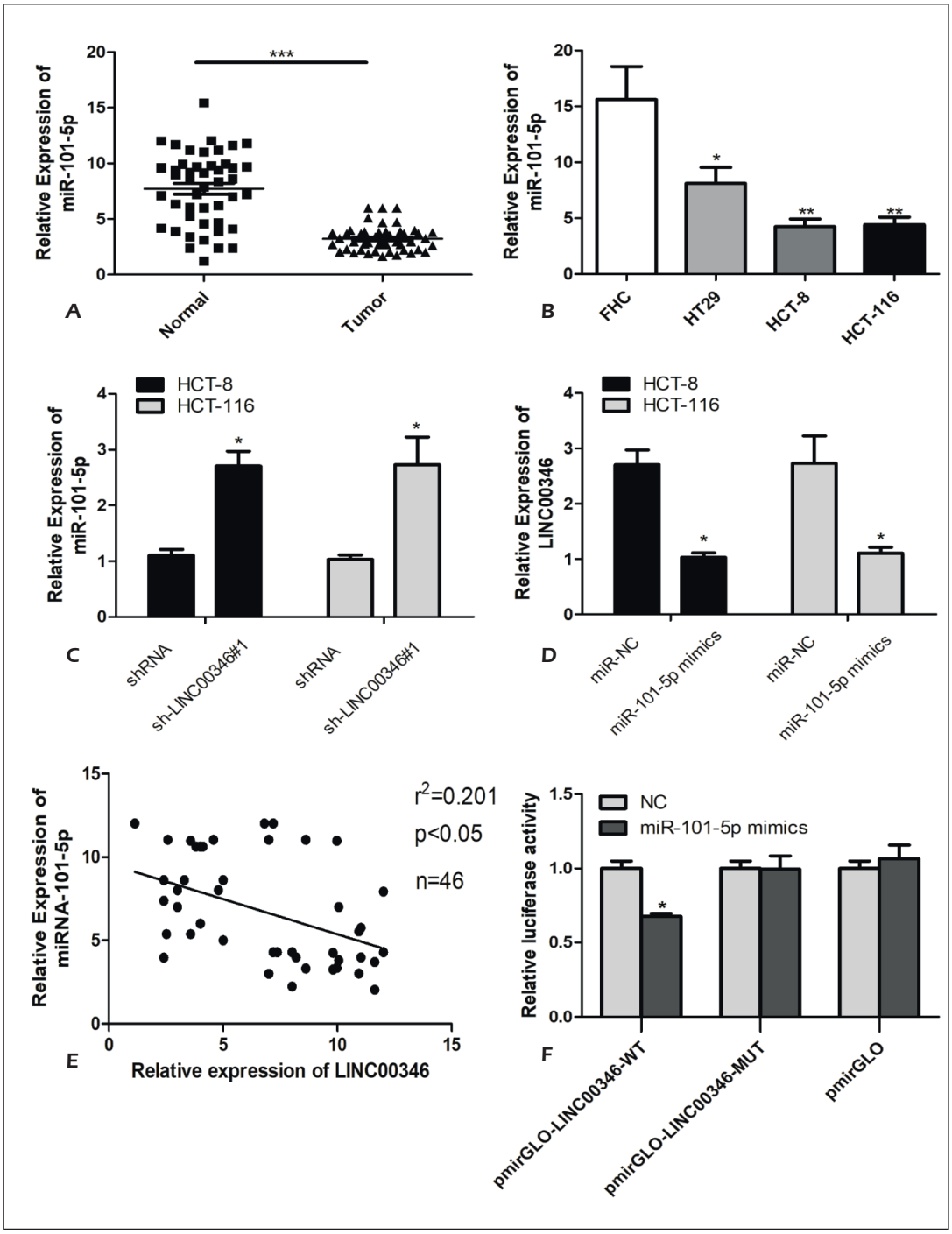

Figure 3. Interaction between LINC00346 and miRNA-101-5p in CRC. **A**, Relative level of miRNA-101-5p in CRC tissues and adjacent normal tissues. **B**, Relative level of miRNA-101-5p in FHC and CRC cell lines. **C**, Relative level of miRNA-101-5p in HCT-8 and HCT-116 cells transfected with shRNA or sh-LINC00346#1. **D**, Relative level of LINC00346 in HCT-8 and HCT-116 cells transfected with miR-NC or miRNA-101-5p mimics. **E**, Correlation between levels of miRNA-101-5p and LINC00346 in CRC tissues (r^2=0.201, p<0.05, n=46). **F**, Relative luciferase activity in cells co-transfected with pmirGLO-LINC00346-WT/pmirGLO-LINC00346-MUT/pmirGLO and miR-NC/miRNA-101-5p mimics.

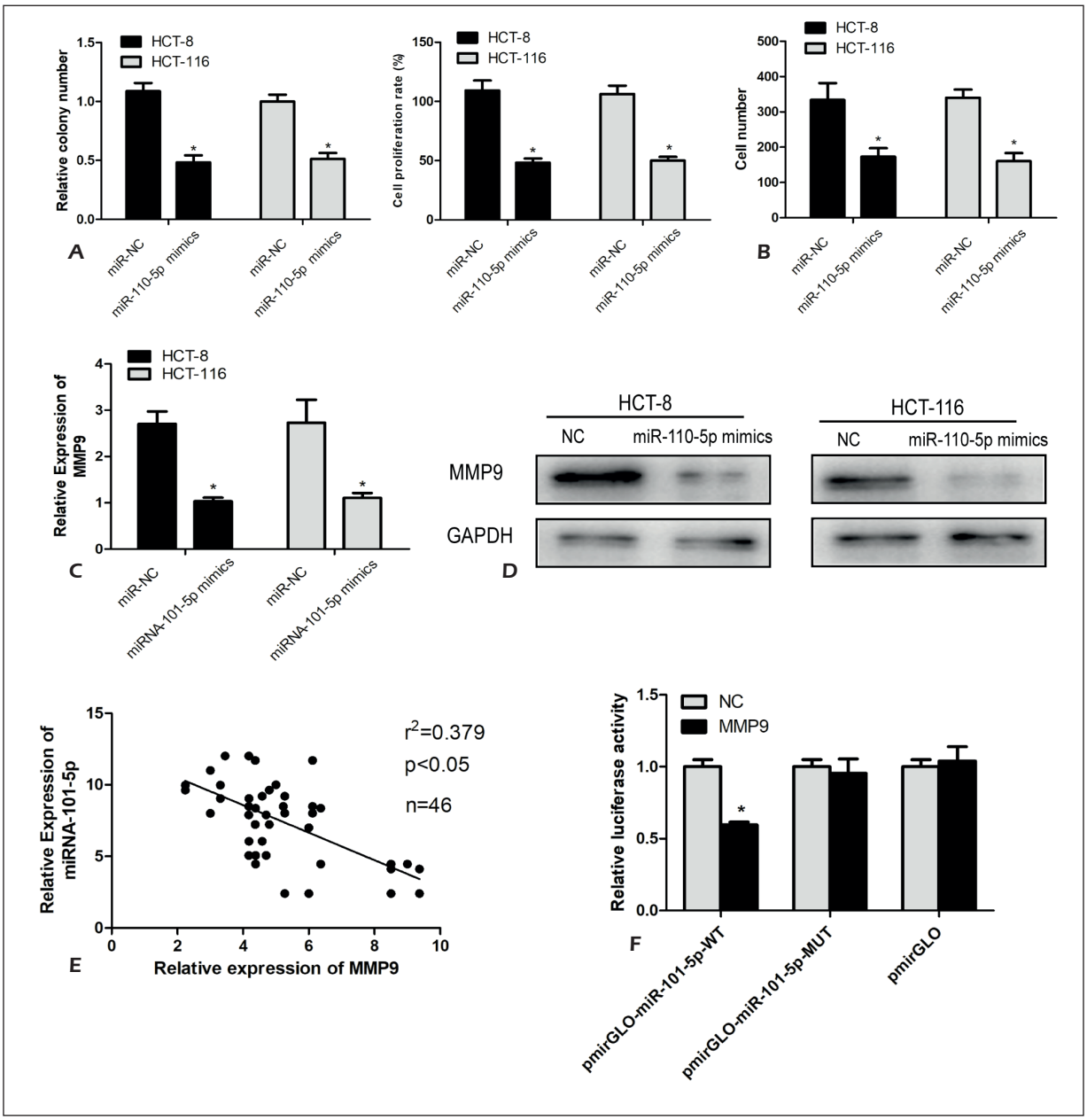

Figure 4. MiRNA-101-5p modulates MMP9 expression in CRC. **A**, Colony formation assay shows the colony number in HCT-8 and HCT-116 cells transfected with miR-NC or miRNA-101-5p mimics. **B**, Transwell assay displays the number of migratory and invasive HCT-8 and HCT-116 cells transfected with miR-NC or miRNA-101-5p mimics. **C**, Relative level of MMP9 in HCT-8 and HCT-116 cells transfected with miR-NC or miRNA-101-5p mimics. **D**, Protein level of MMP9 in HCT-8 and HCT-116 cells transfected with miR-NC or miRNA-101-5p mimics. **E**, Correlation between levels of miRNA-101-5p and MMP9 in CRC tissues (r^2=0.379, p<0.05, n=46). **F**, Relative luciferase activity in cells co-transfected with pmirGLO-miRNA-101-5p-WT/pmirGLO-miRNA-101-5p-MUT/pmirGLO and NC/pcDNA-MMP9.

Through online prediction, binding sequences were identified between LINC00346 and miRNA-101-5p, which were further verified by Dual-Luciferase reporter gene assay. Similarly, MMP9 was uncovered to be the direct downstream of miRNA-101-5p. We demonstrated that LINC00346 promoted the malignant progression of CRC *via* targeting miRNA-101-5p/MMP9 axis, which provided therapeutic targets for CRC treatment.

Conclusions

This study demonstrated that LINC00346 is upregulated in CRC and related to metastasis and poor prognosis of CRC. Besides, it accelerates the malignant progression of CRC *via* targeting miRNA-101-5p/MMP9.

Conflict of Interests

The authors declare that they have no conflict of interests.

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