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LINC00641 induces the malignant progression of colorectal carcinoma through the miRNA-424-5p/PLSCR4 feedback loop

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Abstract. – OBJECTIVE: To illustrate the role of LINC00641 in inducing the malignant progression of colorectal cancer (CRC) through the miRNA-424-5p/PLSCR4 feedback loop.

PATIENTS AND METHODS: LINC00641 levels in paired CRC and non-tumoral tissues were detected by quantitative real-time polymerase chain reaction (qRT-PCR). Its prognostic potential in CRC was assessed by Kaplan-Meier method. Changes in proliferative and migratory abilities of HCT116 and SW620 cells transfected with si-LINC00641 were evaluated by 5-Ethynyl-2'- deoxyuridine (EdU), cell counting kit-8 (CCK-8) and transwell assay. The feedback loop LINC00641/miRNA-424-5p/PLSCR4 was identified through Dual-Luciferase reporter assay and its involvement in CRC progression was finally explored by rescue experiments.

RESULTS: LINC00641 was upregulated in CRC tissues, which was an unfavorable factor to the overall survival of CRC. Proliferative and migratory abilities of HCT116 and SW620 cells were inhibited by knockdown of LINC00641. LINC00641 could competitively bind miRNA-424-5p, thereby abolishing its inhibitory effect on PLSCR4 expression. Knockdown of PLSCR4 could inhibit proliferative and migratory abilities of HCT116 and SW620 cells.

CONCLUSIONS: LINC00641 stimulates proliferative and migratory abilities of CRC through the miRNA-424-5p/PLSCR4 feedback loop.

Key Words:
Colorectal cancer, LINC00641, MiRNA-424-5p, PLSCR4.

Introduction

Colorectal cancer (CRC) originates in the colon, which mainly affects the sigmoid colon. It is the third most-common gastrointestinal tumor[1]. In recent decades, the incidence of CRC has gradually increased in the world. People between 40-50 years are a high risk population for CRC, and the number of male CRC patients is far more than females (2-3:1)[1]. Currently, surgical resection, chemotherapy and immunotherapy are preferred to CRC[2]. Therapeutic efficacy remains low to invasive, metastatic and recurrent CRC cases, leading to a poor prognosis[3,4]. The occurrence and development of CRC involve multiple pathogenic factors. High-fat diet, insufficient dietary fiber and intestinal flora disturbance are the main environmental factors; while familial adenomatous polyposis and familial genetic nonpolyposis are typical genetic factors for the carcinogenesis of CRC[5]. Besides, gene mutations are of significance as well.

Long non-coding RNAs (lncRNAs) consist of about 200-600 nucleotides. They generally do not encode proteins, but regulate gene expressions through transcriptional, epigenetic and post-transcriptional ways[6]. For a long period, lncRNAs are considered as non-functional byproducts of RNA polymerase II transcription[7]. However, recent studies have shown that lncRNAs are involved in many important regulatory processes[8]. Notably, IncRNA can inhibit downstream gene expressions or mediate chromatin remodeling and histone modification. Through binding to specific proteins, their cytoplasmic localization can be altered by lncRNAs. As a structural component, lncRNAs participate in the formation of nucleic acid protein complexes[9-11].

LINC00641 is a functional lncRNA that is relevant to human diseases. It is a prognostic marker for glioblastoma[12]. Through regulating cell autophagy, LINC00641 participates in the onset of disc degeneration[13]. Serving as an anti-cancer

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lncRNA, LINC00641 inhibits bladder cancer progression *via* regulating miR-197-3p[14]. In the present study, we aim to explore the biological function of LINC00641 in CRC progression and the molecular mechanism.

Patients and Methods

Collection of Clinical Specimens

Fifty pairs of CRC and adjacent non-tumoral tissues were collected from CRC patients treated in Shanxi Cancer Hospital from October 2018 to March 2019. The basic characteristics were shown in Table I. Clinical specimens were pathologically confirmed, and their tumor stage was standardly determined based on the criteria of Union for International Cancer Control (UICC). All participants were not treated with radiotherapy and chemotherapy before operation. Other kind of tumor that migrated to the colon was excluded. This investigation was approved by the research Ethics Committee of Shanxi Cancer Hospital and complied with the Helsinki Declaration. Informed consent was obtained from patients.

Cell Culture

CRC cell lines (HCT116, HT-29, SW480, SW620, LoVo) and human intestinal epithelial cell line (NCM460) were provided by the Institute of Hematology, Chinese Academy of Medical Sciences (Beijing, China). Cells were cultivated in Roswell Park Memorial Institute-1640 (RPMI-1640) (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco, Rockville, MD, USA).

Table I. The clinical pathological characteristics of the CRC patients.

Characteristics		No. of case
Gender	Male	28
	Female	22
Age at diagnosis	< 60	14
	≥ 60	36
Differentiation	Poor	21
	Moderately	13
	Well	15
Tumor size	< 5 cm	24
	≥ 5 cm	26
Depth of invasion	T1 + T2	18
	T3 + T4	32
Lymph node status	N0	23
	N1 + N2	27
TNM stage	I + II	27
	III + IV	22

Transfection

Transfection plasmids were synthesized by GenePharma (Shanghai, China). Adherent cells in the 6-well plate were cultured to about 80% density and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 h cell transfection, cells were collected for verifying transfection efficacy and functional experiments.

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

Cells or tissues were lysed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) for isolating RNAs. Qualified RNAs were reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using AMV reverse transcription kit (TaKaRa, Otsu, Shiga, Japan), followed by qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan) at 92°C for 10 min, and 40 cycles at 92°Cfor 10 s and 60°Cfor 1 min. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were served as the internal references. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$. LINC00641: F: 5'-GTAACTCTATGTACAACGTTAA-3', R: 5'-TAGAAGTCAACTCATTATGCTGCTG-3'; miRNA-424-5p: F: 5'-GCCAGCAGCAATTCATGT-3', R: 5'-TATGGTTTTGACGACTGTGTGAT-3'; PLSCR4: F: 5'-CCTTCAGATGCACCTGCTGTTG-3', R: 5'-CCGCAACAAAGCCAATGGTGAC-3'; U6: F: 5′-CTCGCTTCGGCAGCACA-3′, R: 5′-AACGCTTCACGAATTTGCGT-3′; GAPDH: F: 5′-GGAATCCACTGGCGTCTTCA-3′, R: 5′-GGTTCACGCCCATCACAAAC-3′.

Cell Counting Kit-8 (CCK-8) Assay

Cells were inoculated in a 96-well plate with 2×10^3 cells/well. At 6, 24, 48 and 72 h, optical density at 450 nm of each sample was recorded using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) for plotting the viability curves.

Transwell

Transwell chambers (Millipore, Billerica, MA, USA) were inserted in each well of a 24-well plate, where 1×10^4 cells were applied in the upper layer of the chamber, and 600 μL of medium con-

taining 10% FBS was applied in the bottom. After cell culture for 48 h, migratory cells to the bottom were captured following fixation and staining, which were counted in 5 randomly selected fields per sample.

5-Ethynyl-2′- Deoxyuridine (EdU) Assay

Cells were pre-inoculated in a 12-well plate with 5×10^4 cells/well. They were incubated in 4% methanol for 30 min, followed by 10-min permeabilization in 0.5% TritonX-100, and 30-min reaction in 400 μL of 1×ApollorR. Afterwards, cells were dyed in 4',6-diamidino-2-phenylindole (DAPI) for another 30 min. Positive EdU-stained cells were calculated.

Western Blot

Cells were lysed in radioimmunoprecipitation assay (RIPA) (Beyotime, Shanghai, China) for isolating protein samples. The concentration of cellular protein was determined by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). Protein samples were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and loaded on polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was blocked in 5% skim milk for 2 h. They were incubated with primary and secondary antibodies, followed by band exposure.

Dual-Luciferase Reporter Assay

Binding sites between miRNA-424-5p and LINC00641 or PLSCR4 were predicted using online tools, which were used for generating Luciferase vectors. Cells pre-seeded in a 96-well plate (1.5×10^4 cells/well) were co-transfected with Luciferase vectors and miRNA-424-5p mimics or NC. At 48 h, 35 μl of fresh medium and 35 μl of Luciferase substrate were replaced per well for 10-min mixture, followed by measurement of Luciferase activity (Promega, Madison, WI, USA).

Statistical Analysis

Statistical analysis was conducted using Statistical Product and Service Solutions (SPSS) 17.0 (SPSS Inc., Chicago, IL, USA) and figures were depicted by Graph-Pad Prism (Version X; La Jolla, CA, USA). The Student's *t*-test was performed for comparing differences between groups. Pearson correlation test was conducted to assess the correlation between two genes in CRC tissues. Kaplan-Meier curves and log rank test were enrolled for the survival analysis. A significant difference was set at $p<0.05$.

Results

LINC00641 Was Highly Expressed in CRC

In comparison to non-tumoral tissues, LINC00641 was highly expressed in CRC tissues (Figure 1A). Based on the follow-up information of recruited CRC patients, we found that high level of LINC00641 predicted poor overall survival (Figure 1B). It is suggested that LINC00641 may be a promising factor predicting the clinical outcome of CRC. Furthermore, LINC00641 was upregulated in CRC cell lines than that of human intestinal epithelial cell line (Figure 1C). HCT116 and SW620 cells were used to construct LINC00641 knockdown model by transfection of si-LINC00641 (Figure 1D).

LINC00641 Stimulated Proliferative and Migratory Abilities of CRC

As EdU assay uncovered, transfection of si-LINC00641 reduced EdU-positive rate in HCT116 and SW620 cells (Figure 2A, 2B). Consistently, knockdown of LINC00641 decreased viability in CRC cells, indicating that LINC00641 stimulated proliferative ability of CRC (Figure 2C, 2D). Transwell assay showed lower migratory cell number in CRC cells transfected with si-LINC00641 than those transfected with si-NC (Figure 2E, F).

LINC00641 Targeted MiRNA-424-5p and Negatively Regulated its Level

Using bioinformatic tools, a binding site was predicted in miRNA-424-5p 3'UTR that was base-paired to LINC00641 sequences (Figure 3A). Subsequently, wild-type and mutant-type LINC00641 vectors were synthesized for performing Dual-Luciferase reporter assay. Overexpression of miRNA-424-5p remarkably declined Luciferase activity in the wild-type vector, while that in the mutant-type one was not affected (Figure 3B, 3C). It is detected that LINC00641 targeted miRNA-424-5p. Transfection efficacy of miRNA-424-5p inhibitor was examined in HCT116 and SW620 cells (Figure 3D, 3E). Knockdown of miRNA-424-5p upregulated LINC00641 in CRC cells and on the contrary, knockdown of LINC00641 upregulated miRNA-424-5p (Figure 3F, 3G).

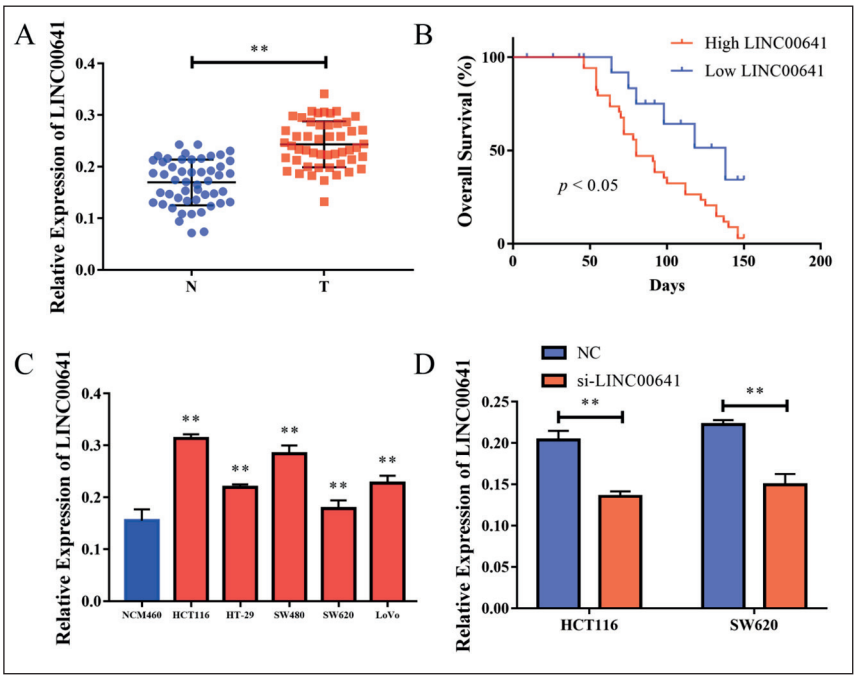

Figure 1. LINC00641 was highly expressed in CRC. **A,** LINC00641 level in non-tumoral and CRC tissues; **B,** Overall survival in CRC patients expressing high or low level of LINC00641; **C,** LINC00641 level in CRC cell lines; **D,** Transfection efficacy of si-LINC00641 in HCT116 and SW620 cells. **p<0.01.

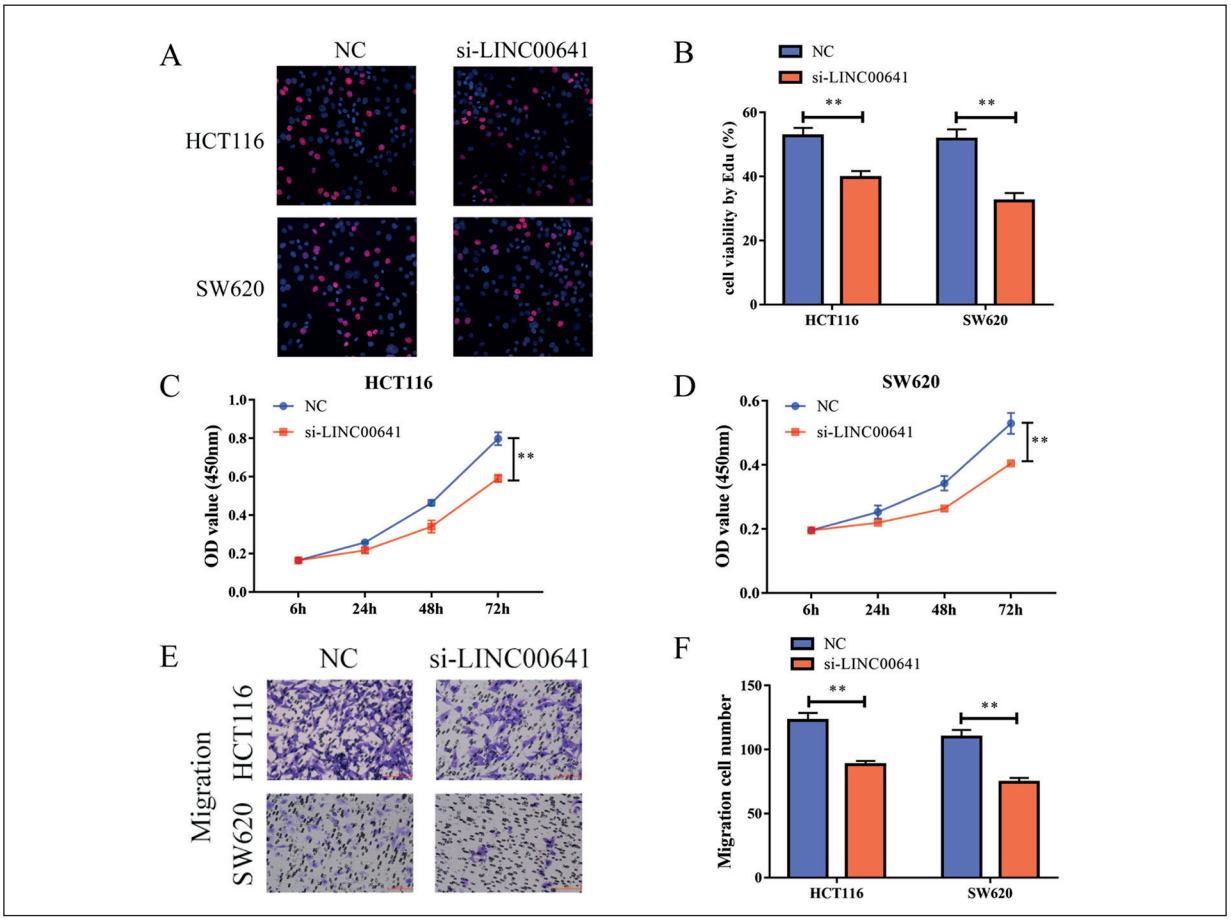

Figure 2. LINC00641 stimulated proliferative and migratory abilities of CRC. **A, B,** EdU-positive rate in HCT116 and SW620 cells with LINC00641 knockdown (magnification 200×); **C, D,** Viability in HCT116 and SW620 cells with LINC00641 knockdown; **E, F,** Migration in HCT116 and SW620 cells with LINC00641 knockdown (magnification 200×). **p<0.01.

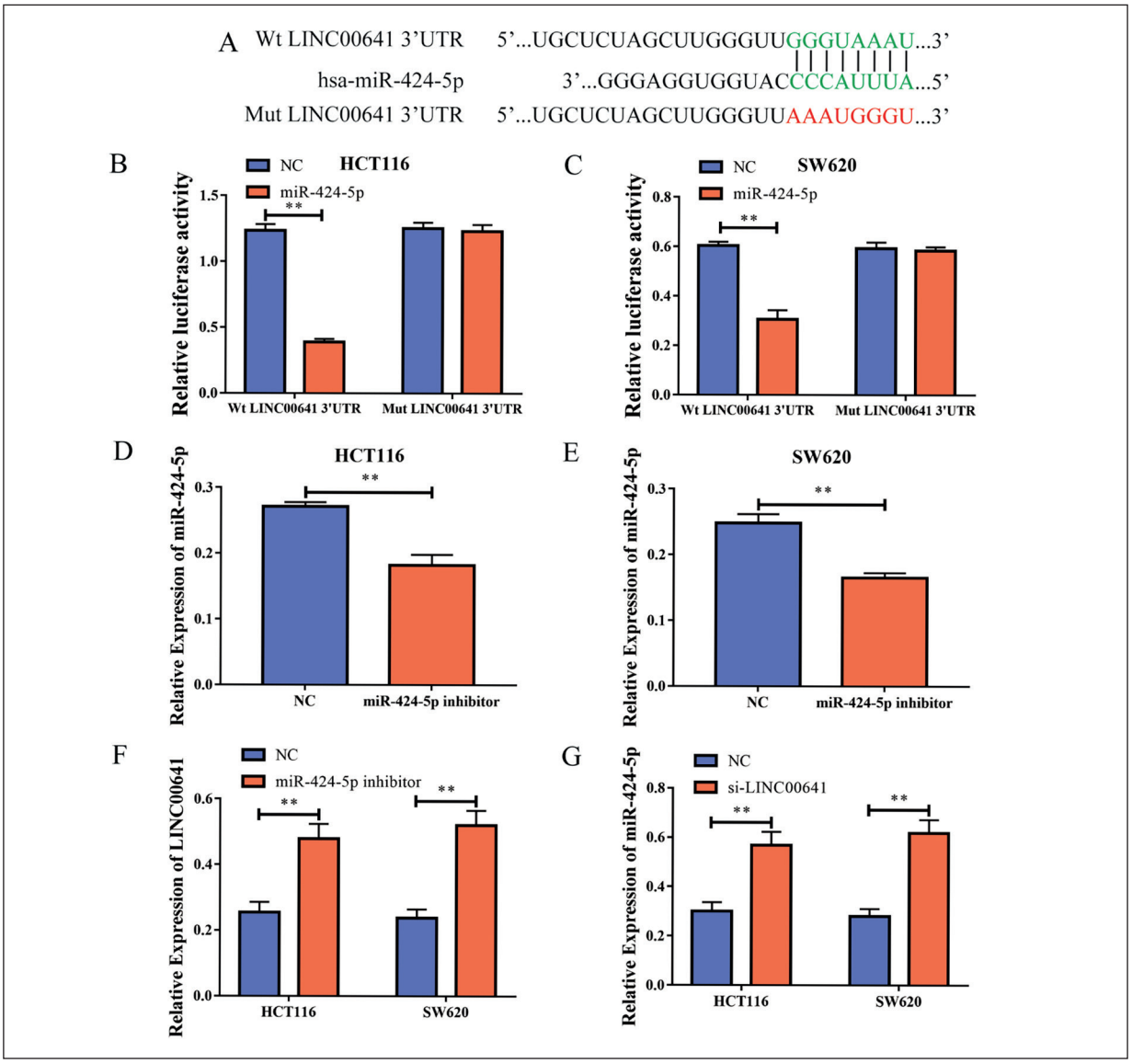

Figure 3. LINC00641 targeted miRNA-424-5p and negatively regulated its level. **A,** Binding site in miRNA-424-5p 3'UTR that was base-paired to LINC00641 sequence; **B, C,** Luciferase activity in LINC00641 vectors affected by miRNA-424-5p; **D, E,** Transfection efficacy of miRNA-424-5p inhibitor in HCT116 and SW620 cells; **F,** LINC00641 level in HCT116 and SW620 cells with miRNA-424-5p knockdown; **G,** MiRNA-424-5p level in HCT116 and SW620 cells with LINC00641 knockdown. $^{**}p<0.01$.

PLSCR4 Was the Target Gene of MiRNA-424-5p

In the same way, we predicted the binding site in PLSCR4 3'UTR that was base-paired to miRNA-424-5p sequences (Figure 4A). Their binding relationship was further indicated by Dual-Luciferase reporter assay (Figure 4B, 4C). To focus on the biological role of PLSCR4, si-PLSCR4 was constructed (Figure 4D). Transfection of si-PLSCR4 markedly upregulated miRNA-424-5p and downregulated LINC00641 in HCT116 and SW620 cells (Figure 4E, 4F). Moreover, knockdown of miRNA-424-5p upregulated PLSCR4, while knockdown of LINC00641 downregulated PLSCR4 in CRC cells (Figure 4G, 4H). In comparison to non-tumoral tissues, PLSCR4 was upregulated in CRC tissues and it was positively correlated to LINC00641 (Figure 4I, 4J).

Knockdown of PLSCR4 Inhibited Proliferative and Migratory Abilitiesof CRC

We next explored the role of PLSCR4 in regulating CRC cell phenotypes. As EdU and CCK-8 assay showed, knockdown of PLSCR4 reduced

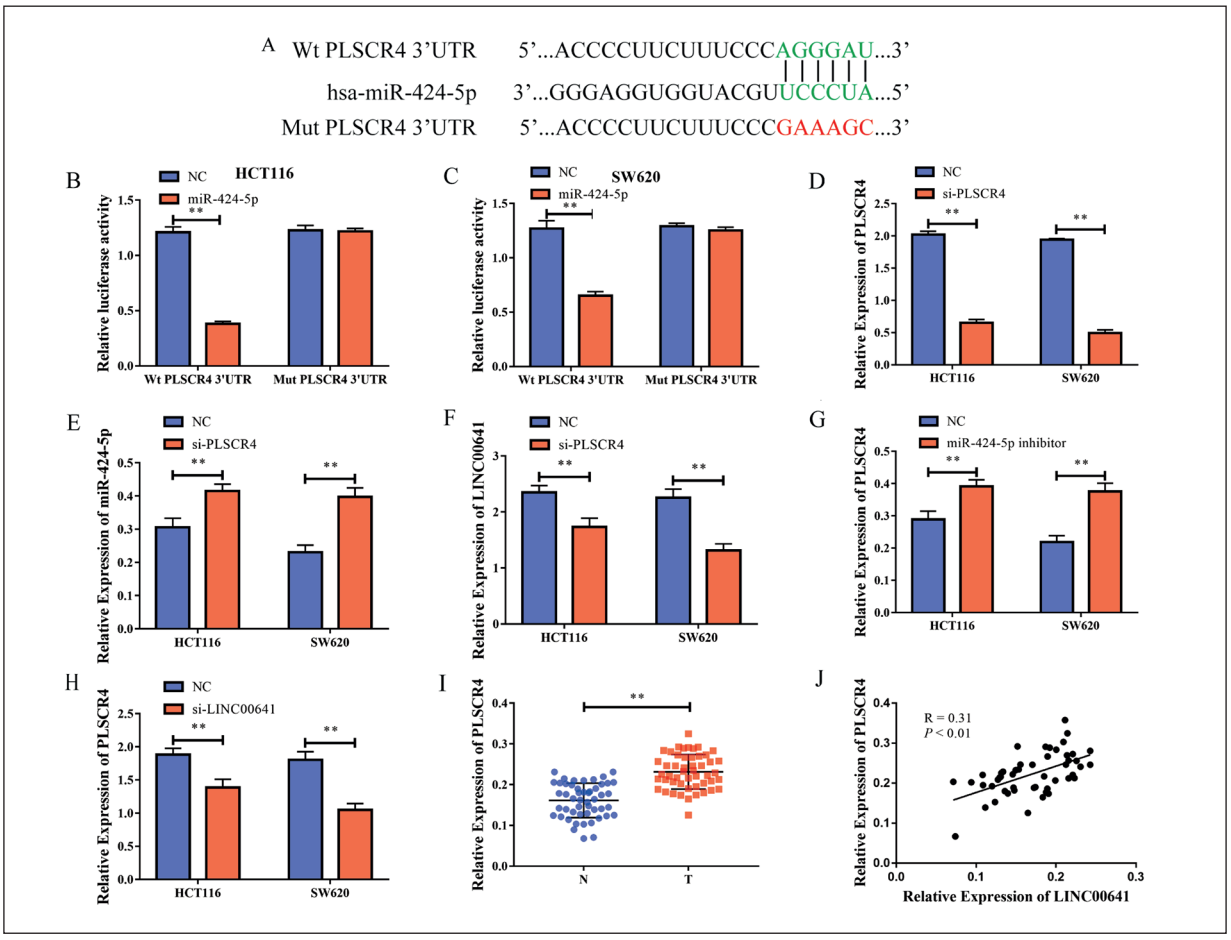

Figure 4. PLSCR4 was the target gene of miRNA-424-5p. **A,** Binding site in PLSCR4 3'UTR that was base-paired to miRNA-424-5p sequence; **B, C,** Luciferase activity in PLSCR4 vectors affected by miRNA-424-5p; **D, E,** Transfection efficacy of si-PLSCR4 in HCT116 and SW620 cells; **F,** LINC00641 level in HCT116 and SW620 cells with PLSCR4 knockdown; **G,** PLSCR4 level in HCT116 and SW620 cells with miRNA-424-5p knockdown; **H,** PLSCR4 level in HCT116 and SW620 cells with LINC00641 knockdown; **I,** PLSCR4 level in non-tumoral and CRC tissues; **J,** A positive correlation between PLSCR4 and LINC00641. $^{**}p<0.01$.

EdU-positive rate and viability in HCT116 and SW620 cells, suggesting the inhibited proliferative potential (Figure 5A-5D). In addition, migratory cell number was declined in CRC cells transfected with si-PLSCR4 (Figure 5E, 5F). PLSCR4 was verified to promote proliferative and migratory abilities of CRC.

Discussion

As the fourth leading cause of cancer deaths in the world, CRC seriously threatens human health[1,15]. The prognosis of CRC is not optimal owing to the high rates of metastasis and recurrence[2]. Therefore, effective biomarkers for screening CRC and predicting its metastasis are of clinical significance. During the past decade, potentials of lncRNAs as tumor biomarkers have been emerged[16-25]. Specifically, a certain number of lncRNAs have been identified as CRC biomarkers[26,27]. Our findings uncovered that LINC00641 was highly expressed in clinical samples of CRC, which was verified to be a promising prognostic factor. Knockdown of LINC00641 remarkably inhibited proliferative and migratory abilities of HCT116 and SW620 cells. We thus speculated that LINC00641 was an oncogene involved in the pathological progression of CRC.

Human phospholipid scramblases (hPLSCRs) are type II membrane proteins, which belong to ATP-independent phospholipid transporters[28]. So far, four isoforms of hPLSCRs (hPLSCR1-4) have been identified, which are highly conserved

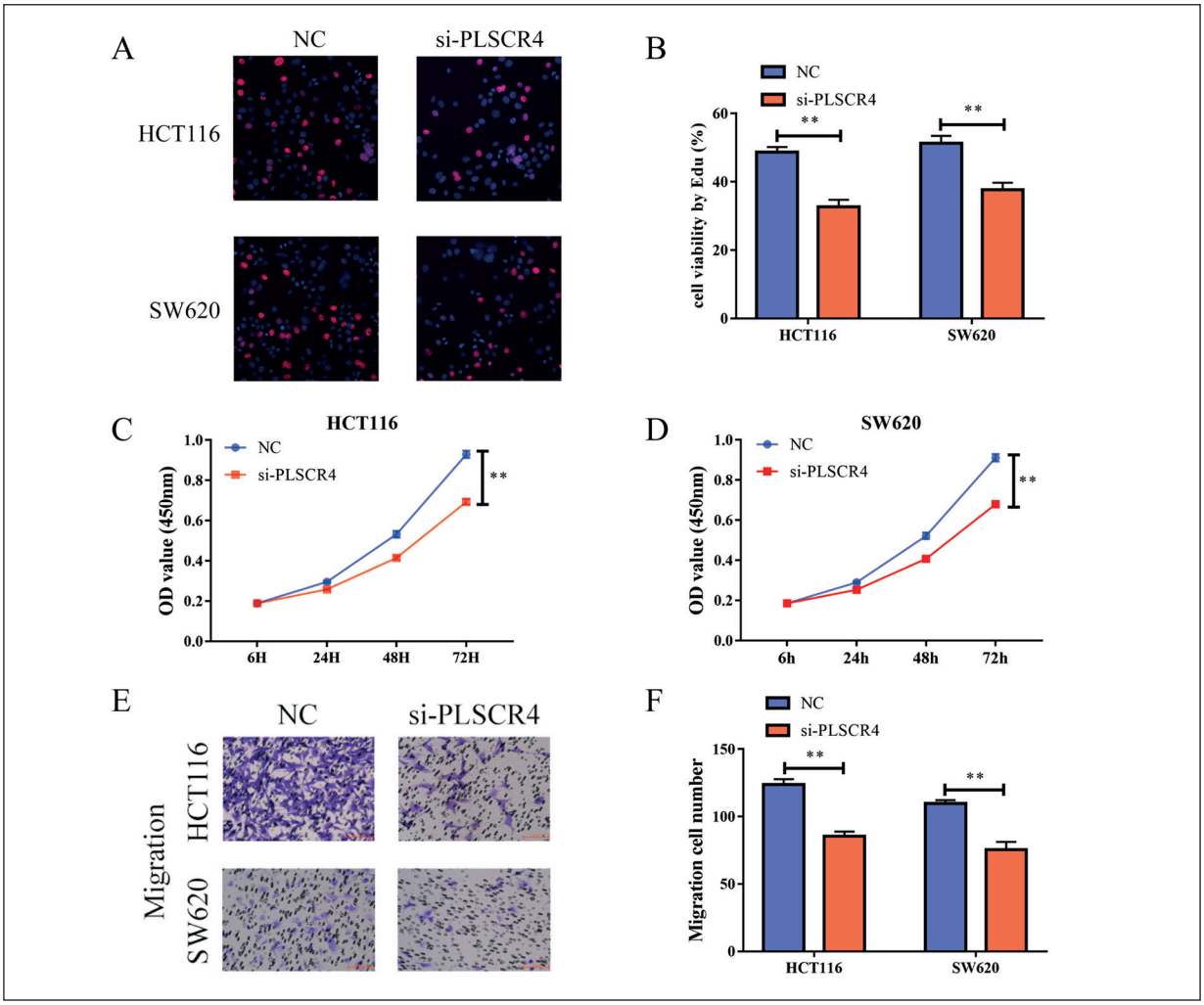

Figure 5. Knockdown of PLSCR4 inhibited proliferative and migratory abilities of CRC. **A, B,** EdU-positive rate in HCT116 and SW620 cells with PLSCR4 knockdown (magnification 200×); **C, D,** Viability in HCT116 and SW620 cells with PLSCR4 knockdown; **E, F,** Migration in HCT116 and SW620 cells with PLSCR4 knockdown (magnification 200×). $^{**}p<0.01$.

from *Caenorhabditis (C.)* elegans to humans[29]. hPLSCR1 is the most widely studied isoform. It is responded to increased cytosolic Ca^{2+} concentration and thus induces transbilayer migration of phospholipid transporters non-specifically and bidirectionally[30]. Cell activation, injury and apoptosis are able to trigger the activation of hPLSCR1[31]. The deduced amino acid sequences of hPLSCR2-4 have about 59%, 47% and 46% homology with hPLSCR1, respectively. Sequence alignment revealed the existence of conserved domains in the members of the disordered enzyme family, including cysteine-rich domains and Ca^{2+}-bound EF-hand domains[32]. The palmitoylation of hPLSCR1 contributes to its localization on the plasma membrane (PM), while the mutation of the palmitoylation motif causes nuclear localization of all expressed proteins[33]. It is reported that both hPLSCR1 and hPLSCR4 are localized in the nucleus through the importin α/β pathway[34]. Cytokines and interferon alpha are capable of regulating hPLSCR1 expression[35]. hPLSCR4 has been shown to interact with CD4 on the PM, and their interaction is regulated by secreted leukocyte protease inhibitor (SLP1)[36]. Acting as a transcriptional regulator of IP3R, hPLSCR1 enhances the degrading activity of human topoisomerase IIα in the nucleus[37].

In the previous researches, LINC00641 was reported as a ceRNA for miR-378a to regulate ZBTB20 expression in acute myeloid leukemia[38]. Further, LINC00461 could also act as a scaffold

for miR-942 to affect the survival of patients with renal cell carcinoma[39]. In our research, based on the findings of bioinformatic analysis and Dual-Luciferase reporter assay, LINC00641/miRNA-424-5p/PLSCR4 feedback loop was identified. In detail, LINC00641 could competitively bind miRNA-424-5p, thereby abolishing its inhibitory effect on PLSCR4 expression. PLSCR4 was upregulated in CRC tissues and positively correlated to LINC00641 level. Notably, knockdown of PLSCR4 attenuated proliferative and migratory abilities of CRC cells *in vitro*. Taken together, LINC00641 stimulated the progression of CRC by the miRNA-424-5p/PLSCR4 feedback loop. In the present research, we firstly reported the role and mechanism of LINC00461 in CRC and may provide a new target for therapy in the future.

Conclusions

Summarily, this study demonstrated that LINC00641 stimulates proliferative and migratory abilities of CRC through the miRNA-424-5p/PLSCR4 feedback loop.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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