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Long noncoding RNA SLC30A10 promotes colorectal tumor proliferation and migration *via* miR-21c/APC axis

L. HOU[1], P. LIU[1], T. ZHU[2]

[1]Department of Gastrointestinal Surgery, Affiliated Hospital of Chengde Medical College, Chengde, China
[2]Department of Oncology, Affiliated Hospital of Chengde Medical College, Chengde, China

Abstract. – OBJECTIVE: Colorectal cancer is a common malignancy and a common cause of tumor-related death. Long non-coding RNAs (lncRNAs) have become an important regulatory factor and tissue specific biomarker for a variety of cancers, including colorectal cancer. Recent evidence indicates that the novel lncRNA SLC30A10 plays an important role in tumor progression and metastasis. However, its role and molecular mechanisms in colorectal cancer are unclear.

PATIENTS AND METHODS: SLC30A10 expression was detected in 12 colorectal cancer and adjacent normal tissues by quantitative reverse transcription PCR. Insights into the underlying mechanisms of competitive endogenous RNAs (ceRNAs) were determined by transwell assay, CCK8 assay, and luciferase assay.

RESULTS: SLC30A10 was down-regulated in colorectal cancer tissues and cell lines, and its low expression was positively correlated with colorectal cancer progression and metastasis. Functionally, SLC30A10 depletion promotes cell proliferation and migration in colorectal cancer cells, while SLC30A10 overexpression has the opposite effect. Bioinformatics prediction and luciferase assay indicated that miR-21c is a direct target of SLC30A10, which plays the role of ceRNA in regulating colorectal cancer metastasis. In addition, miR-21c specifically targets APC gene.

CONCLUSIONS: Our findings suggest that reduced expression of SLC30A10 is associated with aggressive tumor phenotypes and poor patient outcomes in colorectal cancer. SLC30A10 inhibits colorectal cancer progression and metastasis by acting as a ceRNA for miR-21c to regulate APC expression, suggesting that SLC30A10 may serve as a potential prognostic biomarker and anti-metastatic therapeutic target for colorectal cancer.

Key Words:
Colorectal cancer, SLC30A10, MiR-21c/APC, Proliferation, Migration.

Introduction

Colorectal cancer is a common malignant tumor of the digestive tract that occurs in the colorectal[1]. Nowadays, colorectal cancer has become a common type of cancer because of its global incidence and mortality rate, which are among the highest[3]. In 2018, the number of new cases reached about 2 million, and nearly 900,000 people lost their life because of colorectal cancer[3,4]. Despite the improvement in diagnosis and treatment options, the incidence of the disease has been greatly reduced, but the five-year survival rate of colorectal cancer cells is still not satisfactory in recent years[5]. Therefore, it is important to further reveal novel diagnostic and therapeutic methods, as well as investigate the underlying molecular mechanism of the metastasis and invasion of colorectal tumor.

Much of the research in proliferation and invasion of colorectal cancer cells in the last years has related to protein-coding genes[6]; in the meanwhile, a growing number of studies have shown that non-coding RNA also plays a crucial role in the physiological function of colorectal cancer cells[7]. LncRNA is a type of non-coding RNA named for its transcription length of more than 200 nucleotides and lacks protein coding ability, plays an important role in many life activities such as epigenetic regulation, cell cycle regulation and cell differentiation regulation[8-11]. The pattern of mutual regulation between lncRNA and miRNA and its downstream target genes is closely related to the occurrence and development of tumors[12-14]. miRNA acts as an important post-transcriptional regulation factor and its activity can be regulated by lncRNA adsorption by "sponge". This lncRNA is also called competitive endogenous RNA (ceRNA)[15,16]. LncRNA competes as a ceRNA to bind with miRNA, thus regulating the protein level of

Corresponding Author: Lei Hou, MD; e-mail: hbcdcyfyhoulei@163.com

the encoded gene and participating in the regulation of the biological behavior of the cell[17,18].

To date, little is known about the role of SLC30A10 in the metastasis and invasion of colorectal cancer. In this study, we aimed to explore the biological roles of SLC30A10 in colorectal cancer, as well as to illustrate the molecular mechanisms. We first detected the SLC30A10 levels in colorectal cancer tissues and SW480 cell line. Then, the proliferation, migration and invasion abilities of SW480 miR-21c cells were measured after transfection of lentiviral SLC30A10. Finally, we assessed the regulatory relationship between SLC30A10 and miR-21c, miR-21c and APC, respectively, and found that SLC30A10 could bind with miR-21c to further regulate the expression of APC. Our study uncovered a critical role of lncRNA-SLC30A10 in colorectal cancer progression, which plays a key role in the progression of colorectal cancer by regulating the inhibitory effect of miR-21c on APC as ceRNA.

Patients and Methods

Patients and Tumor Samples

In this study, we used NCCN clinical practice guidelines for patient diagnosis. 12 cases of colorectal cancer patients were diagnosis by colonoscopy. 12 pairs of colorectal cancer tissues and adjacent normal tissues were collected from surgically treated and pathologically diagnosed colorectal cancer cases and then stored at -80°C. Besides, we also conducted a case review about the TNM stages and the presence of lymph node metastasis of these 12 colorectal cancer patients. Patient information was included in Table I. No significant differences in the 12 pairs of samples in terms of diagnostic indicators and prognostic factors were found. This study was approved by the Ethics Committee of our Institute. Patients and their families had been fully informed that their specimens would be used for scientific research, and all participating patients had signed informed consent.

Table I. Demographic data.

Gender	Male	Female
Patients numbers	7	5
BMI (kg/m²) ± SD	20.7±5.3	22.1±5.0
Age (years)		
< 45	3	3
≥ 45	4	2
TNM stage		
I–II	3	2
III–IV	4	3
Lymph node metastasis		
Negative	2	2
Positive	5	3

All the patients were selected randomly.

Cell Culture

Human colorectal cancer cells SW480 cells were purchased from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Rockville, MD, USA) supplied with 10% FBS (Gibco, Rockville, MD, USA) and 1% penicillin-streptomycin (Gibco, Rockville, MD, USA) and incubated at 37°C in an atmosphere of 5% CO_2.

Construction of Lentivirus and Cell Transfection

Lentiviral Lnc-SLC30A10 and Lnc-SLC30A10 shRNA were synthesized and constructed by Shanghai GenePharma Co., Ltd (Shanghai, China). For miR analysis, the miR-21c mimic, miR-21c inhibitor and the negative control were constructed by Shanghai GenePharma Co., Ltd (Shanghai, China). And to knock down APC, si- APC plasma and negative control plasma were constructed by Shanghai GenePharma Co., Ltd (Shanghai, China). For transfection, 1×10^4 cells were seeded in 6-well plates and cultured with Dulbecco's Modified Eagle Medium (DMEM). Lipofectamine 2000 kit (Invitrogen, Carlsbad, CA, USA) and Opti-MEM® I reduced serum medium were used for transfection. For analysis of Lnc- SLC30A10, cells were transfected with Lnc- SLC30A10 shRNA (referred as to sh) and negative control shRNA (referred as to nc), respectively. For analysis of miR-21c, cells were transfected with miR-21c inhibitor, and control cells were transfected with empty vector, respectively. The cells without transfection were used as the control (referred as to control). After cultures were incubated for 30 min, they were replaced with DMEM containing 10% FBS. Then, at indicated time point after transfection, cells were harvested for further study.

Transwell Assay

To test the migration ability of SW480 cells, transwell plates with a pore size of 8 μm (Millipore Inc, Billerica, MA, USA) were used to conduct transwell assay. SW480 cells were treated differently and the lower chamber was added with DMEM supplemented with 20% FBS. The upper side of the membrane

was wiped with a cotton swab to remove the cells that did not migrate, and cell numbers in five random fields were counted in each sample.

RNA Extraction and qRT-PCR

The culture plates were taken out, and the cells were washed with PBS. After treatment, total RNA of cells was extracted by using TRIzol reagent (Life Technologies, Waltham, MA, USA) according to the manufacturer's instructions. Samples were stored at room temperature for 30 min. The reverse transcription of cDNA was performed with a PrimeScript™ RT reagent Kit (TaKaRa, Otsu, Shiga, Japan) according to the manufacturer's instructions. For qRT-PCR, PCR primers were synthesized by GenePharma (ShangHai Gene Pharma, Shang-Hai, China) and sequences were listed in Table II. SYBR Premix Ex Taq II (TaKaRa, Otsu, Shiga, Japan) was used to detect the expression.

CCK8 Assay

The CCK-8 kit (Dojindo, Molecular Technologies, Kumamoto, Japan) was used to measure the cells proliferation according to the manufacturers' instructions. In brief, 5×10^3 cells were seeded in 96-well plates uniformly. After treated with regulated medium, the medium was removed, and cells were washed with PBS solution for 3 times. Then CCK8 dilution was added to the 96-well plates and incubated at 37°C in an atmosphere of 5% CO_2 for 2 hours. After incubation, the plates were taken out, and cell proliferation was measured using multi-detection microplate reader. The absorbance (OD) value at 490 nm of each well was detected.

Luciferase Assay

After transfection for 48 h, the luciferase activities were measured by using the dual luciferase reporter assay system (Promega, Madison, WI, USA) according to the manufacturer's protocol. Renilla luciferase activities were normalized to the firefly luciferase activities and the data were expressed as the fold change relative to the corresponding control groups which were defined as 1.0.

Statistical Analysis

Unless otherwise indicated, all data are processed by Statistical Product and Service Solutions (SPSS) 16.0 statistical software (SPSS Inc., Chicago, IL, USA). Each assay was applied at least three independent experiments or replicates. All data were presented as mean ± SD. Student's *t*-test, one-way analysis of variance (ANOVA) and multiple comparison between the groups were performed by using SNK method, in which $*p < 0.05$, $**p < 0.01$ represented as the difference significance.

Results

SLC30A10 and APC Were Downregulated in Human Colorectal Cancer Tissues and Cells

To figure out the roles of SLC30A10 and APC in colorectal cancer progression, we performed qRT-PCR to observe the expressions of SLC30A10 and APC in colorectal cancer tissues and adjacent normal tissues at first. The results showed that not only the expressions of SLC30A10 were significantly downregulated in colorectal cancer tissues compared with that of adjacent normal tissues, but also APC produced a similar phenomenon (Figure 1A and 1B). To further explain the biological function of SLC30A10 in colorectal cancer, we performed qRT-PCR to detect SLC30A10 expression in human colorectal cancer cell lines SW480. The results showed that the expressions of SLC30A10 and APC were both significantly downregulated in colorectal cancer cells compared with human epithelia cells HEK293 cells ($p < 0.05$) (Figure 1C and 1D).

Migration and Invasion of Colorectal Cancer Cells Were Significantly Inhibited after the SLC30A10 Expression was Upregulated

To explore the function of SLC30A10 in the progression of colorectal cancer, we did two experiments. One is to construct SLC30A10 (Lnc-SLC30A10) overexpressing lentivirus and

Table II. Primer sequences for qRT-PCR

Genes	Forward	Reverse	Tm (°C)
SLC30A10	5'-GGTTAACCCATCGAATTGGC-3'	5'-GATTAGGAAACCGGATGAA-3'	60
miR-21c	5'-GGATCCAGGGATAGAGGTACA-3'	5'-GGTACGGCCTACATTCCTG-3'	61
APC	5'-GAAATGCTTACCTAACAGC-3'	5'-GTTCGGGAGGACCAGCCAAT-3'	62
GAPDH	5'-ACCCTTTGGACGCACGGCAT-3'	5'-GGTTCAGTCCCTGACGTACCG-3'	62

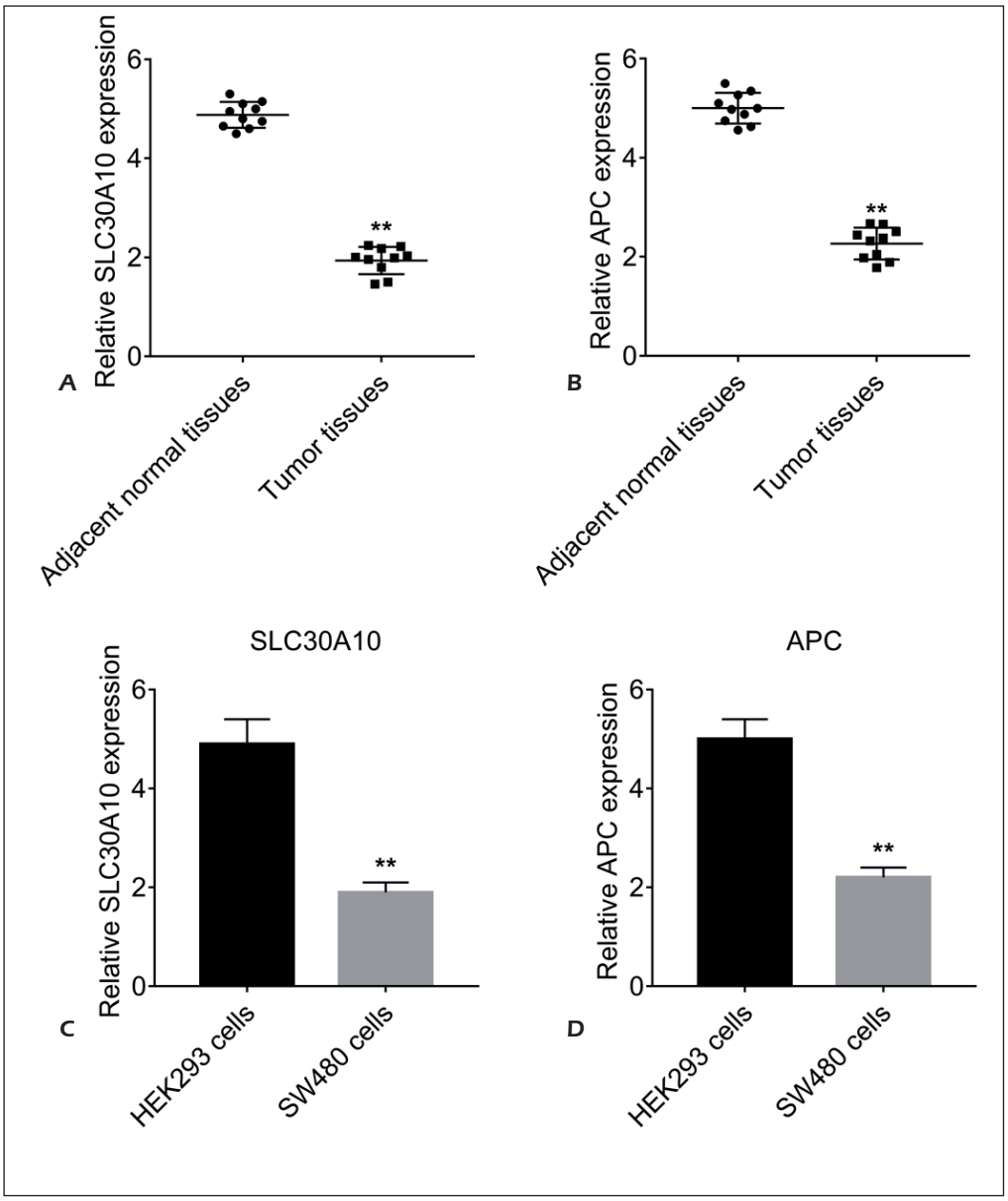

Figure 1. SLC30A10 and APC were downregulated in human colorectal cancer tissues and cells. Relative mRNA expression levels of (**A**) SLC30A10 and (**B**) APC in colorectal cancer tissues and adjacent normal tissues. Relative mRNA expression levels of (**C**) SLC30A10 and (**D**) APC in human colorectal cancer cell line SW480 and HEK293 cells. The data in the figures represent the averages ± SD. Statistically significant differences between the treatment and control groups are indicated as * ($p < 0.05$) or ** ($p < 0.01$).

transfect it into SW480 cells. The other is the synthesis of small interfering RNA (si-SLC30A10) for SLC30A10 and transfection into SW480 cells. Subsequently, the expression of SLC30A10 was detected by qRT-PCR. The results showed that the expression of SLC30A10 was significantly increased in the Lnc-SLC30A10 group compared with the vehicle vector1 ($p < 0.05$), while the expression level of SLC30A10 was decreased in the si-SLC30A10 group compared with the negative control vector2 ($p < 0.05$) (Figure 2A and 2B). To verify the role of SLC30A10 in cell proliferation, CCK8 analysis was performed on SW480 cells after regulation of SLC30A10 expression. The results showed that overexpression of SLC30A10 significantly reduced the proliferation of colorectal cancer cells compared with the control group within 3 days, while inhibition of SLC30A10 expression significantly increased the proliferation of colorectal cancer cells (Figure 2C, 2D). The results suggest that

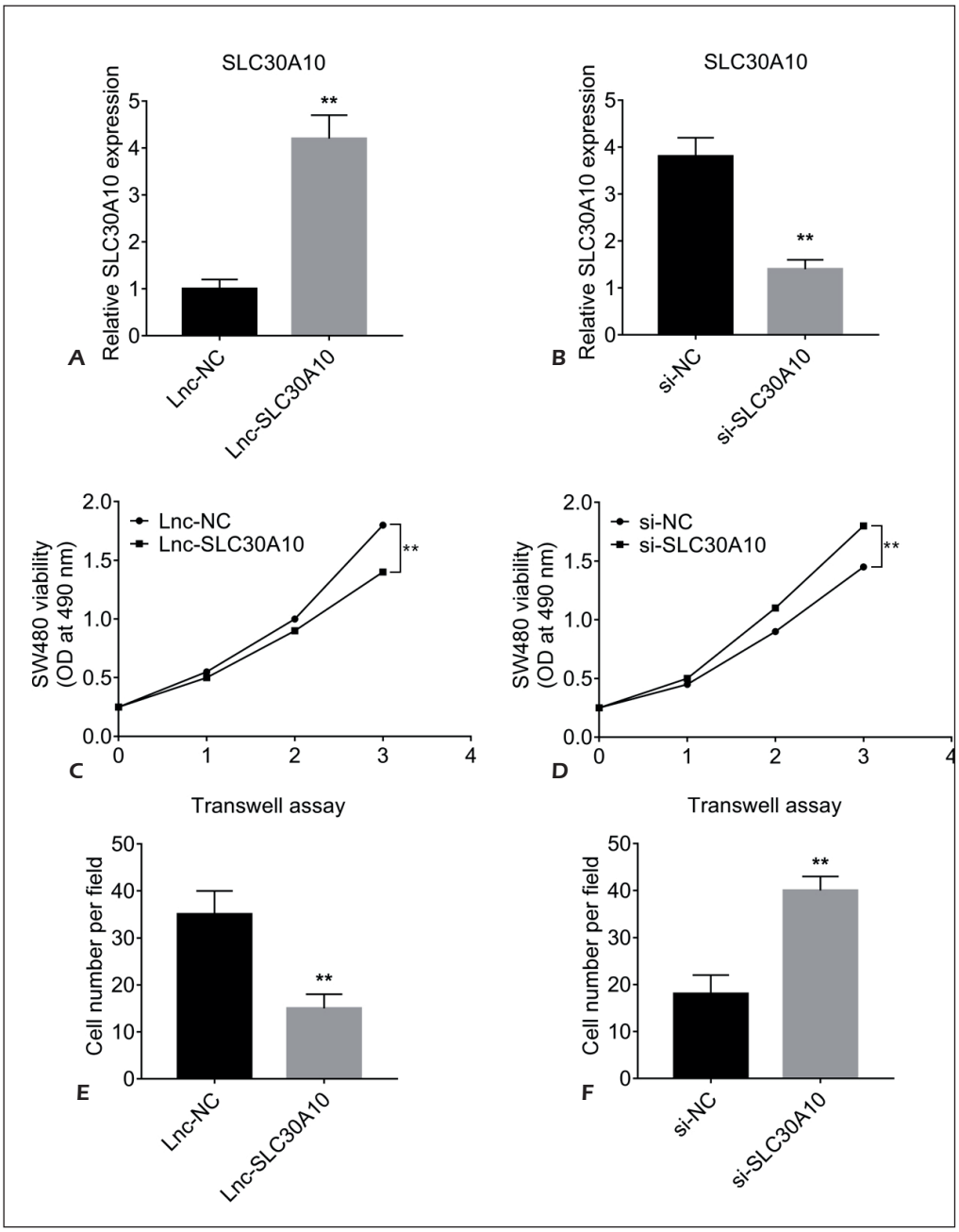

Figure 2. The migration and invasion of colorectal cancer cells were significantly inhibited after upregulated the SLC30A10 expression. **A**, Relative mRNA expression levels of SLC30A10 in SW480 cells transfected with SLC30A10 overexpressing lentiviral (Lnc-SLC30A10) and Lnc-NC. **B**, Relative mRNA expression levels of SLC30A10 in SW480 cells transfected with si-NC and si-SLC30A10. **C**, Absorption at 490 nm of SW480 cells treated with Lnc-SLC30A10 and Lnc-NC revealed by CCK-8 assay at 1 d, 2 d and 3 d. **D**, Absorption at 490 nm of SW480 cells treated with si-SLC30A10 and si-NC detected by CCK-8 assay at 1 d, 2 d and 3 d. The data in the figures represent the averages ± SD. Statistically significant differences between the treatment and control groups are indicated as * ($p < 0.05$) or ** ($p < 0.01$).

changes in the expression of SLC30A10 may affect the proliferation of colorectal cancer cells. To further determine that SLC30A10 affects migration and invasion of colorectal cancer cells, we used transwell analysis to detect the ability of human colorectal cancer cells to migrate after SLC30A10 expression. The results showed that compared with the control group, when SLC30A10 was up-regulated, the response to fetal bovine serum (FBS) and the migration of colorectal cancer cells through the

transwell chamber were significantly reduced (Figure 2E); when SLC30A10 was down-regulated, the response to fetal bovine serum and colorectal cancer cells passed. The migration of transwell chambers has increased significantly (Figure 2F). In summary, the changes of SLC30A10 regulate the migration and invasion of human colorectal cancer cells. By upregulating SLC30A10, the migration and invasion of colorectal cancer cells can be effectively inhibited, and the migration and invasion of colorectal cancer cells can be effectively promoted by downregulation.

miR-21c Expression Was Upregulated in Colorectal Cancer Cells and Negatively Correlated with SLC30A10

To investigate the relevance of SLC30A10 to miRNA, we used StarBase 2.0 to predict the target miRNA of SLC30A10 and found that miR-21c is one of the target miRNAs of SLC30A10. We used qRT-PCR analysis to detect the miR-21c expressions of human colorectal cancer tissues and SW480 cells. The results showed that miR-21c was highly expressed in colorectal cancer tissues compared to adjacent normal tissues and was upregulated in SW480 cells compared to HEK293 cells (Figure 3A and 3B). Then, we used a correlation analysis to further explore the relationship between SLC30A10 and miR-21c. The results showed that miR-21c was significantly negatively correlated with SLC30A10, suggesting that miR-21c may be regulated by SLC30A10 (Figure 3C). From these results, we can observe that miR-21c is highly expressed in colorectal cancer tissues and SW480 cell lines, and negatively correlated with SLC30A10. It has been previously suggested that LncRNAs can act as a competing sponge in regulating the biological functions of miRNAs.

SLC30A10 Can Sponge with miR-21c and Inhibit its Expression in Colorectal Cancer Cells

It has been previously suggested that LncRNAs can act as a competing sponge in regulating the biological functions of miRNAs. From the previous conclusion, we know that miR-21c is negatively correlated with SLC30A10. Therefore, we can assume that the interaction between SLC30A10 and miR-21c is a way to regulate the migration and invasion of colorectal cancer. To validate this hypothesis, we synthesized the SLC30A10-wt luciferase reporter vector and the SLC30A10-mut 3'UTR luciferase reporter vector and assayed for the luciferase reporter gene (Figure 4A). Compared with the normal control, the luciferase activity of SW480 cells that co-transfected with wide type SLC30A10 (SLC30A10-wt) and miR-21c mimic was significantly decreased ($p < 0.01$), and it was

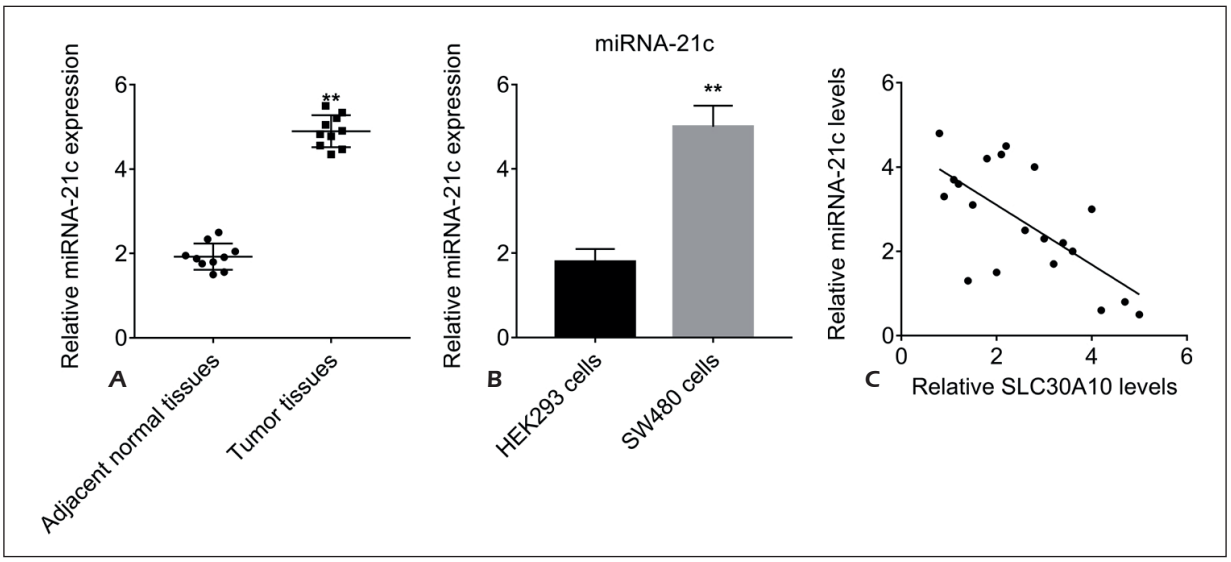

Figure 3. miR-21c expression was upregulated in colorectal cancer cells and negatively correlated with SLC30A10. **A**, Relative miR-21c expression in colorectal cancer tissues and adjacent normal tissues detected by qRT-PCR. **B**, Relative miR-21c expression in SW480 cells and HEK293 cells detected by qRT-PCR. **C**, Correlation analysis was performed to evaluate the relationship between miR-21c and SLC30A10. The data in the figures represent the averages ± SD. Statistically significant differences between the treatment and control groups are indicated as * ($p < 0.05$) or ** ($p < 0.01$).

reversely increased after mutation at the binding site of SLC30A10 (SLC30A10-mut) compared with SLC30A10-wt ($p < 0.01$) (Figure 4B). The results indicate that SLC30A10 can directly bind to miR-21c. In addition, when SLC30A10 is overexpressed, inhibition of miR-21c expression is inhibited, and when SLC30A10 expression is inhibited, miR-21c expression is reversely promoted in SW480 cells (Figure 4C, 4D). We also transfected miR-21c mimics and miR-21c inhibitors into SW480 cells, which showed that miR-21c mimics inhibited SLC30A10 expression, whereas miR-21c inhibitors increased SLC30A10 expression (Figure 4E, 4F). All of these results indicate that miR-21c binds directly to SLC30A10 at the recognition site.

SLC30A10 Served as ceRNA for miR-21c to Further Modulate the Expression of APC

APC is a tumor suppressor gene whose mutation or deletion is closely related to the occurrence of various cancers. To investigate whether miR-21c interacts with APC, we performed qRT-PCR analysis for APC in the presence of miR-21c mimics or inhibitors. After treatment of SW480 cells with the miR-21c mimics, we observed a decrease in APC expression, indicating that miR-21c can downregulate APC expression (Figure 5A). To validate this mechanism, we cloned mice APC 3'-UTR into a luciferase reporter vector and constructed a miR-21c binding mutant in which the putative miR-21C binding site AUGC in APC 3'-UTR the mutation was UACG (Figure 5B). As expected, the dual luciferase reporter results indicated that the miR-21c mimetic significantly down-regulated APC expression, while the point mutation in the APC 3'-UTR abolished the inhibition of miR-21c (Figure 5C). Then, we further verified whether SLC30A10 can regulate the expression of APC by acting with the sponge of miR-21c. The results indicate that SLC30A10 can significantly increase the expression of APC, but the mutation of the binding site of miR-21c and SLC30A10 is effec-

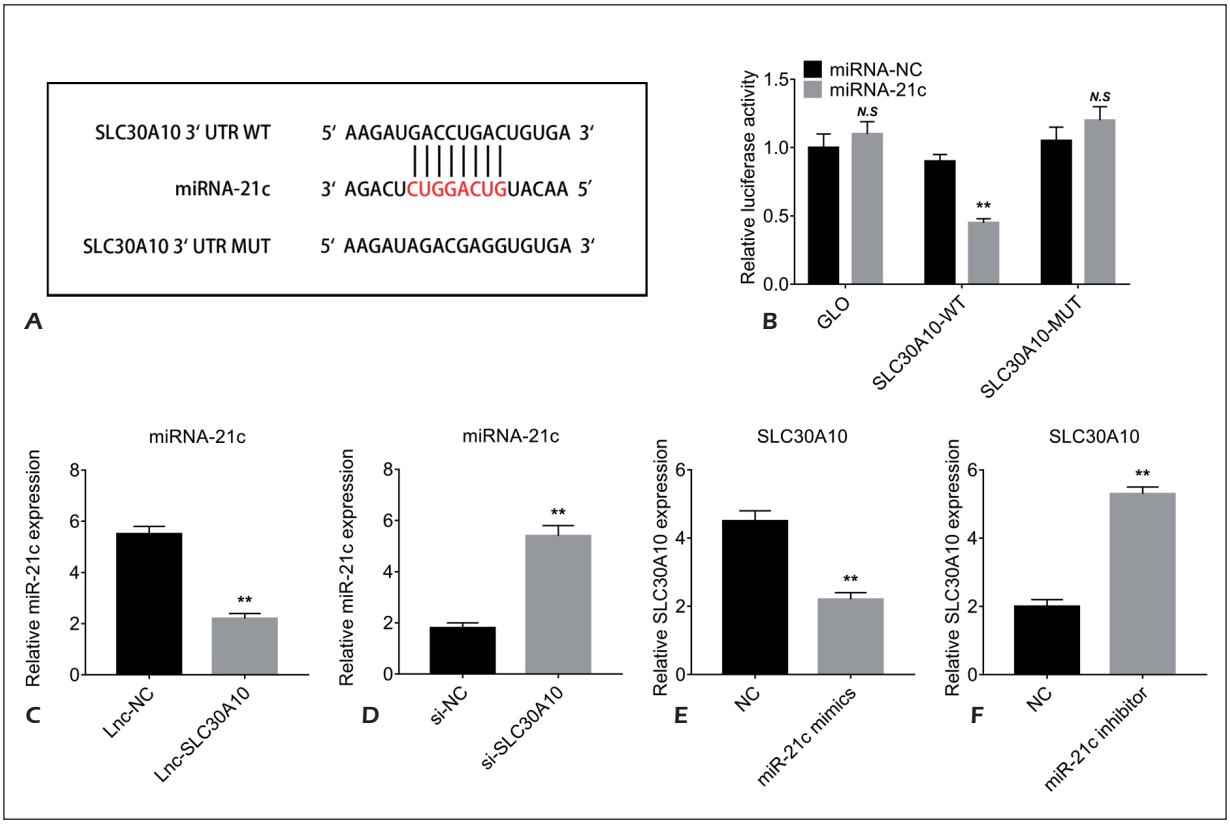

Figure 4. SLC30A10 can sponge with miR-21c and inhibit its expression in colorectal cancer cells. **A**, Sche¬matic illustration of the predicted miR-21c binding sites and mu¬tant sites in SLC30A10. **B**, Relative luciferase activity of SW480 cells. **C-D**, qRT-PCR analysis of miR-21c expression level in SW480 cells transfected with lentiviral SLC30A10 and si-SLC30A10. **E-F**, Relative SLC30A10 expression was detected in SW480 cells after treated with miR-21c mimics and miR-21c inhibitor by qRT-PCR. The data in the figures represent the averages ± SD. Statistically significant differences between the treatment and control groups are indicated as * ($p < 0.05$) or ** ($p < 0.01$).

tively eliminated (Figure 5D). On the contrary, inhibition of miR-21c overcomes the inhibitory effect of SLC30A10 knockdown on APC (Figure 5E). In summary, this data analysis showed that SLC30A10 served as ceRNA for miR-21c to further modulate the expression of APC.

Discussion

Colorectal cancer is a common malignant tumor in China, and its incidence is on the rise[19]. Standard treatments for colorectal cancer still have side effects and resistance[20]. With the continuous development of therapeutic methods, targeted therapy has become an effective treatment for cancer, and lncRNA plays an important role in targeted therapy[21,22].

LncRNA generally refers to non-coding RNAs greater than 200 nucleotides (nt)[23]. Compared with other non-coding RNAs, lncRNA has the characteristics of "three more", that is, many types, many modes of action, and a large number[24,25]. LncRNA is widely involved in many important regulatory processes such as chromosome silencing, genomic imprinting, chromatin modification, transcrip-

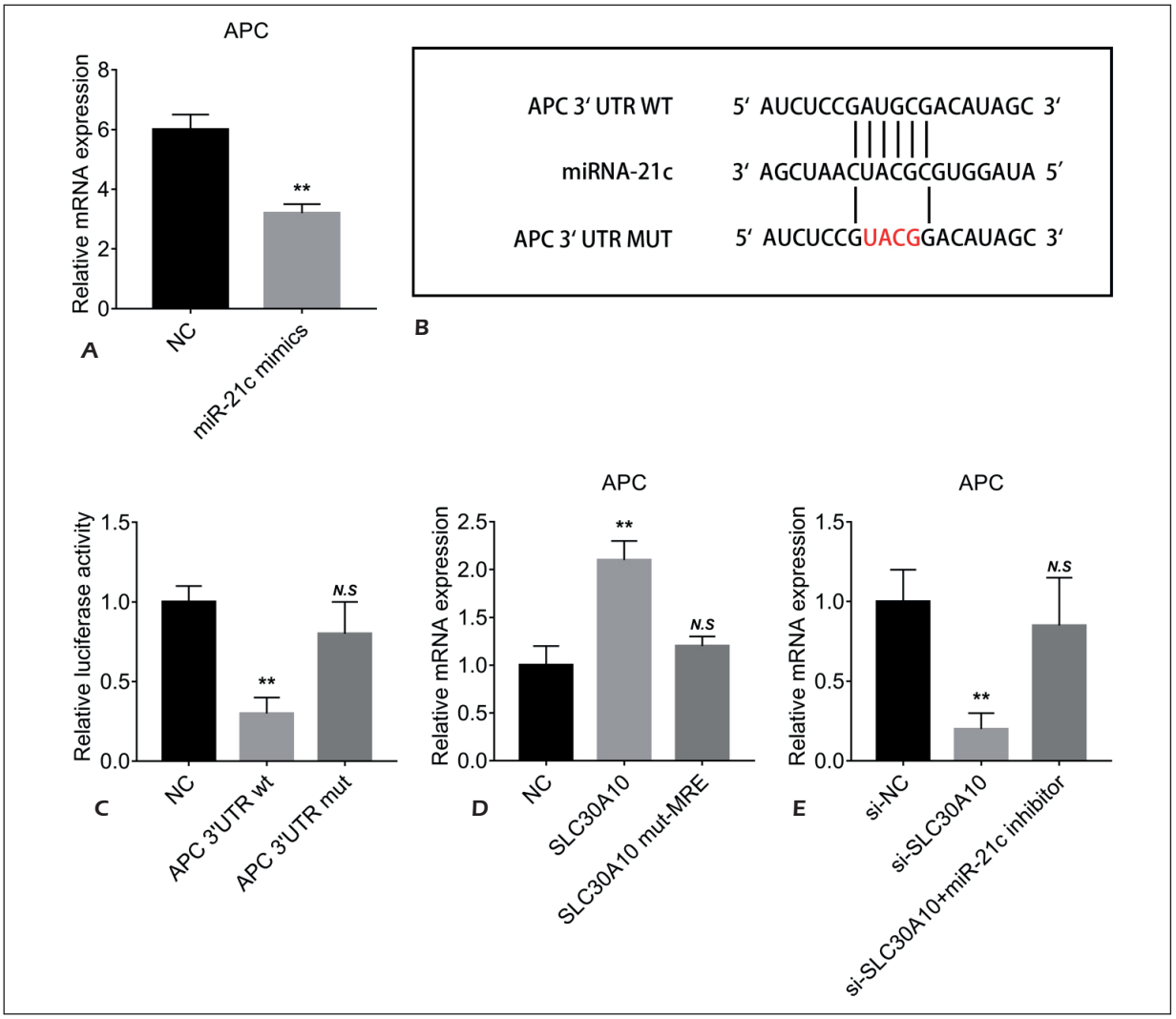

Figure 5. SLC30A10 served as a molecular sponge for miR-21c to further modulate the expression of APC. **A**, qRT-PCR analysis of APC mRNA expression level in SW480 cells treated with the miR-21c mimics. **B**, Schematic illustration of the predicted APC binding sites and mutant sites in miR-21c. **C**, Relative luciferase activity of SW480 cells. **D**, Relative mRNA expression levels of APC in SW480 cells transfected with SLC30A10 and SLC30A10 mut-MRE. **E**, Relative mRNA expression levels of APC in SW480 cells transfected with si-SLC30A10, si-SLC30A10 and miR-21c inhibitor by qRT-PCR analysis. The data in the figures represent the averages ± SD. Statistically significant differences between the treatment and control groups are indicated as * ($p < 0.05$) or ** ($p < 0.01$).

tional activation, transcriptional interference, and nuclear transport[26,28]. In recent years, there was a growing recognition for the functional relevance of lncRNAs in a variety of cancers[29-35]. However, the detailed molecular mechanisms of lncRNAs have not been fully elucidated. Increasing evidence supported the importance of the regulatory mechanism of ceRNAs in tumorigenesis of cancers. Competing endogenous RNAs (ceRNAs) are defined as lncRNAs that act as molecular sponges to compete with the miRNAs in binding to mRNAs[17,36]. This phenomenon resulted in the repression of the activities of miRNAs and the promotion of the expression of mRNA targets[15,37].

In this research, a lncRNA named SLC30A10 with lower expressed was found in colorectal cancer cells. Based on the ceRNA hypothesis, we hypothesized that lncRNA-SLC30A10 can act as an endogenous bait for miRNAs, thereby affecting the binding of miRNAs to their mRNA targets. The results indicate that SLC30A10 binds directly to miR-21c and acts as a miR-21c sponge in colorectal cells (Report by dual luciferase). Simultaneously, in this study we detected that miR-21c can regulate the expression of tumor suppressor gene APC. As a key tumor suppressor gene, APC is not only found in most colorectal cancers, but also in other cancers such as liver cancer; besides, mutations in the APC gene may lead to colorectal cancer. Therefore, this study finally wants to explain that one of the causes of metastasis and invasion of colorectal cancer in cancer cells is due to the low expression of APC, which is due to the high expression of miR-21c, and the high expression of miR-21c is caused by the low expression of LncRNA-SLC30A10.

SLC30A10 has been reported to play a crucial role in regulating thyroxine production, as well as for the excretion of manganese by hepatocytes and intestinal cells[38,39]. However, the regulatory role of SLC30A10 in tumor development, especially in colorectal cancer, has not been reported.

Conclusions

This study revealed the regulatory role of SLC30A10 in colorectal cancer, which served as a ceRNA of miR-21c to upregulate suppressor gene APC expression and inhibit metastasis and invasion of colorectal tumor.

Conflict of Interests

The authors declare no conflict of interest

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