A specific Upregulated IncRNA in Colorectal Cancer Promotes Cancer Progression by Modulating miR-185-5p/CCND2 Axis

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Research

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Abstract

Background

Adenomatous polyposis coli (APC) gene mutations were found in most colorectal cancer patients and functioned as an important inducer of tumorigenesis. Long non-coding RNA (lncRNA) plays a crucial role in the pathogenesis of various diseases, including colorectal cancer (CRC). Here we investigated the role of SURC which is specific upregulated in CRC progression.

Methods

Based on the previous microarray results, weighted correlation network analysis (WGCNA) and lncRNA-mRNA co-expression network analysis were used to identify a lncRNA (SURC) and found it was specific up-regulated in CRC patients by qPCR and FISH staining. Chromatin immunoprecipitation (ChIP) assay was used to demonstrate the regulatory effect and mechanism of APC mutation on SURC expression. The effects of SURC on proliferation and cell cycle were determined by *in vitro* and *in vivo* experiments. Chromatin Isolation by RNA Purification (CHIRP) and luciferase reporter assay were carried out to illustrate the interaction between SURC, miR-185-5p and CCND2.

Results

We found that SURC was specific up-regulated in CRC, but not in other solid tumor, when compared with normal adjacent tissues. High expression of SURC correlates with poorer disease-free survival and overall survival of CRC patients. Mutated APC protein resulted in stabilization of β -catenin in CRC, which promotes the transcription of SURC via binding to its promoter. Knockdown of SURC impaired CRC cell proliferation, colony formation and CRC tumor growth. Mechanistically, after transcription, SURC was transferred to cytoplasm and inhibits miR-185-5p expression via binding to miR-185-5p and inhibiting the synthesis of miR-185-5p from pri-miR-185-5p, which results in CCND2 expression.

Conclusion

Collectively, these results indicated that SURC promoted CRC tumor growth via interacting with miR-185-5p and regulating the activity of miR-185-5p/CCND2 axis which would be a novel diagnosis and prognosis prediction target for CRC.

Background

Colorectal cancer (CRC) is the third most common cancer in the world^[1]. It's estimated that there were more than 1.8 million new cases of CRC and 881,000 deaths of CRC in 2018^[2]. The incidence of CRC is closely related to adenomatous polyposis coli (APC) gene mutation, which act as a tumor suppressor gene^[3]. APC regulates the expression of Wnt target genes via promoting the degradation of β -Catenin^[4]. The protein encoded by wild-type APC gene promotes the degradation of β -Catenin by binding with β -Catenin protein. Deletion or mutation of APC gene leads to the accumulation of β -Catenin in the nucleus,

which activates canonical Wnt signaling pathway^[5]. APC gene mutations were found in familial adenomatous polyposis (FAP) and 70-80% of sporadic CRC patients have APC gene inactivation^[6]. Thus, understanding the mechanism under APC regulating the carcinogenesis and progression of CRC is necessary and of clinical significance.

Long non-coding RNA (lncRNA) is a class of non-coding RNA (ncRNA) with a length of more than 200 bp and without the ability to encode proteins^[7]. Recent studies have revealed that lncRNAs play a regulatory role in the progression of malignant cancers, such as breast cancer, gastric cancer, lung cancer and CRC^[8-11]. In CRC, a series of lncRNAs was demonstrated as tumor suppressors and tumor promoters, which regulate gene expression and participate in the occurrence and development of CRC by regulating cell proliferation, cell cycle, epithelial-to-mesenchymal transition (EMT), drug resistance and metastasis^[12]. LncRNA-SNHG11 promote the invasion and metastasis of CRC cells by interacting with HIF-1 α and activating the expression of HIF-1 α ^[13]. LncRNA-RPPH1 is up-regulated in tissues of CRC patients and induce CRC progression by inducing EMT process^[14]. LINC01106 activates the expression of Gli family factors and drive the growth and stemness of CRC^[15]. Current research indicates that an APC-activated lncRNA-APC1 regulates the progression of CRC via non-canonical Wnt signaling^[16]. Recent years, a large number of lncRNAs have been identified by using bioinformatic and high-throughout methods^[17]. However, there are still a large number of lncRNAs with unknown functions, which need further research to determine new diagnostic and therapeutic targets.

Based on the previous microarray results of AOM/DSS induced CAC model, we used weighted correlation network analysis (WGCNA) and lncRNA-mRNA co-expression network analysis to classify and predict the functions and signaling pathways of lncRNAs^[18]. We screened a lncRNA (lncRNA-AK028845) and found it was specific up-regulated in CRC, thus named it as specific up-regulated lncRNA in CRC (SURC). In the present study, we determined the expression and correlation with prognosis of SURC in CRC and investigated the underlying mechanism that mediated the specific up-regulation of SURC in CRC. Then the function of SURC in CRC was explored, followed with the investigation of molecular mechanism under the function. The findings would provide a novel perceive for understanding the role of APC mutation in CRC progression and supply a novel target for CRC diagnosis and prognosis prediction.

Materials And Methods

Clinical Specimens

The experiments were approved by West China Hospital of Sichuan University Biomedical Research Ethics Committee (Number: 2018(280)). Clinical samples were collected between 2013 and 2014 at the Department of gastrointestinal Surgery, West China Hospital, Sichuan University. All patients were histologically diagnosed with cancer and underwent surgery, followed up regularly after operation. All the frozen tissues were fixed with 4% paraformaldehyde, embedded with paraffin and cut into 4 μ m slides for H&E staining. The histopathological evaluation was performed by one experienced pathologist and

reviewed by other two pathologists. The qualified samples were used for tissue microarray by Outdo Biotech (Shanghai, China), and the tissue microarray was named as HXCRC cohort. The disease-free survive and overall survive were recorded by arranging follow-up in the next five years after surgery.

Animal experiment

In vivo experiments were performed in compliance with Sichuan University guidelines concerning animal use and care. Female BALB/c nude mice (4-5 weeks) were purchased from Beijing HFK Bioscience Co. (Beijing, China). All mice were growth in a pathogen-free conditions with free access to water and food. For tumor xenograft experiments, 5×10^6 SW620, LoVo and RKO cells were injected subcutaneously into the ventral subcutaneous of mice. At 10 days post cell injection, the tumor length and width were measured by vernier caliper every three days. The tumor volume were calculated as: tumor volume = length $\times$ width² $\times$ 0.52. After the subcutaneous tumor grew well, the mice were euthanized and the tumors were dissected and weighed.

Cell culture and treatment

SW620, 293T, HCoEpiC and RKO cells were cultured in Dulbecco's Modified Eagle Medium (Invitrogen, USA) containing 10% fetal bovine serum (FBS, Millipore, Germany) and 1% penicillin/streptomycin. F-12K Nutrient Mixture (Invitrogen, USA) was used to cultivated LoVo cells with 10% fetal bovine serum and 1% penicillin/streptomycin. All cells were purchased from American Type Culture Collection (ATCC, USA) and were detected by the mycoplasma detecting kit (Yise Medical Technology, China) to confirm a non-mycoplasma condition. And all these cells were cultured in the condition of 37°C and 5% CO₂. The small interfering RNAs used in this study were siDicer-1, siDicer-2, siDicer-3 which were purchased from RiboBio (Guangzhou, China). The lentivirus based shSURC-575, shSURC-1083, shAPC (GenePharma, China) were used to infect SW620 and LoVo cell lines. The overexpression plasmid of SURC and APC were designed and purchased from GeneCopoeia (Guangzhou, China). Also, miR-185-5p inhibitor and miR-185-5p mimic were purchased from RiboBio (Guangzhou, China). Actinomycin D was purchased from MedChemExpress (New Jersey, USA), while PNU-74654 and SKL2001 were purchased from Selleck (Texas, USA).

RNA Extraction and Quantification RT-qPCR

TRIzol reagent (Thermo Fisher Scientific, USA) was used to obtain lysates of tissues and cell lines. Then, Chloroform was added and mixed, and was centrifuged at 4 °C for 15 min. Isopropanol and Alcohol were added gradually and centrifuged. After that, we poured out the supernatant, dried the ethanol and added appropriate amount of RNase free water. The purity and concentration of RNA were detected by nanodrop 2000 Ultradifferential photometer. Total 1 µg of RNA was reverse transcribed into cDNA by using PrimeScriptTMRT reagent Kit (Takara, Japan) according to the manufacturer's protocol. Then, SYBR Green Master Mix (Takara, Japan) was used to perform a qPCR reaction with the procedure of 95°C for 600s followed by 42 cycles of 95°C for 5s and 58°C for 30s. The expression levels of GAPDH, β -actin, and U6 snRNA were used as normalizer in vivo and vitro experiments. The relative level of gene expression was

calculated by using the $2^{-\Delta\Delta Ct}$ formula. The sequences of primers used in the present study were supplied in the supplementary data.

CCK8, colony formation

The cultured cells were digested by trypsin and planted in 96-well plates (2000 cells/well). For CCK8 analysis, CCK8 reagent (DoJinDo, China) was diluted in the proportion of 1:10 and added into the cells cultured in 96-well plates at 37 °C for 4 h. After that, the absorbance was detected at a wavelength of 450 nm by using a microplate spectrophotometer. For colony formation experiment, the cultured cells were seeded into 6-well plates (2000 cells/well) for 7-10 days. Then, the cells in the plates were fixed with 4% paraformaldehyde and stained with crystal violet (Beyotime, China). The number of colonies was counted by Image J software.

BrdU assays

The cells were cultivated in 6-well plates (1×10^5 cells/well) and incubated with BrdU (Merck, Germany) at 37°C for 30 min. Then, cells were covered completely with cold 70% ethanol for 5 min. 1.5M HCl was added and incubated for 30 min at room temperature. After that, blocking buffer was added for 1 hour and primary antibody was incubated overnight at 4°C. The cells were incubated with fluorochrome-conjugated secondary antibody for 2 hours at room temperature in dark. Finally, the cells were stained by 4,6-diamidino-2-phenylindole (DAPI, Beyotime, China) and counted under fluorescence microscopy.

Flow cytometry for cell cycle

A cell cycle detection kit (Beytime, China) was used to carry out cell cycle experiment according to the manufacturer's instructions. First, the cells were collected in centrifugal tubes and fixed in 70% ethanol at 4 °C for 24h. After that, the cells were incubated with propidium iodide and RNase for 30 minutes. Finally, a flow cytometer was used to analyse cell cycle distribution.

Western blotting

Protein lysates were extracted from the cells and tissues using RIPA lysis buffer (Beyotime, China) supplemented with Phosphatase Inhibitor Cocktail (Roche, Switzerland) and a phosphatase inhibitor (Roche, Switzerland). Then, a BCA assay kit (Thermo Fisher Scientific, USA) was used to quantify the protein concentrations according to manufacturer's recommendations. After that, 10 µg of total protein lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to polyvinylidene difluoride (PVDF) membranes (Invitrogen, USA) for 60 min at 100V. Membranes were blocked in 5% non-fat milk powder for 2 hours and then incubated with anti-CCND2 (1:1000, Cell Signaling Technology, #3741S), anti-GAPDH (1:10000, ABclonal, AC002) or anti-β-catenin (1:10000, ABclonal, AC026) overnight at 4 °C. After primary antibody incubation, the membranes were washed and incubated with anti-mouse (1:5000, ZSGB-BIP, ZB-2305) or anti-rabbit (1:5000, ZSGB-BIP, ZB-2301) horseradish peroxidase (HRP)-conjugated secondary antibodies for one hour. After the

treatment of ECL substrate (Thermo Fisher Scientific, USA), the blotting images were captured by iBright™ CL1000 Instrument (Invitrogen, USA).

Immunohistochemistry

Mouse and human tissues were embedded in paraffin and cut into 5 µm slices. Paraffin embedded slices were dewaxed and rehydrated in xylene and alcohol. After that, the slices were boiled in 0.01 M citrate buffer (pH 6.0) for 5min. Then, 3% hydrogen peroxide was performed to eliminate endogenous peroxidase for 15 min, and subsequent treatment with goat serum for 20 min at room temperature. Slices were incubated with anti-CCND2 (1:100, Cell Signaling Technology, #3741S) and anti-ki67 (1:100, Abcam, ab16667), antibodies overnight at 4 °C. After being treated with the secondary antibody and horseradish peroxidase (HRP), the DAB staining (MXB Biotechnologies, China) was used for immunohistochemical detection.

Fluorescence in situ Hybridization

Cells were fixed with 4% paraformaldehyde at room temperature for 10 min and permeabilized with penetrant solution (PBS containing 0.5% Triton X-100) at 4 °C for 5 min. After that, probes were added for hybridization at 37 °C overnight. After DAPI staining, images were obtained with fluorescence microscope. FISH probes and lncRNA FISH Kits were designed and purchased by RiboBio (Guangzhou, China).

Tissues were fixed overnight in 4% paraformaldehyde and cut into 5µm slices. The tissue sections were immersed in xylene and dewaxed twice at room temperature for 20 minutes and placed in 100% ethanol, 85% ethanol and 70% ethanol for two minutes. Then, the sections were boiled in H₂O for 3 min. After that, the tissue sections were digested in pepsin (Merck, Germany) solution for 30 min, immersed in 0.2M HCl solution for 10 min, and dehydrated in gradient ethanol. The probe was added for hybridization at 83 °C for 5 minutes and 42 °C overnight. The sections were washed, dyed with DAPI and observed under fluorescence microscope.

ChIRP assay

Beads used in this paper were purchased from Invitrogen and the ChIRP experiment was performed according to the protocol. In a brief, cells were collected and crosslinked with 1% glutaraldehyde solution to maintain the interaction of RNA-chromatin. The cells were lysed and the lysates were treated with ultrasonic crushing so that most of DNAs were interrupted into 100-500 bp in length. The probe purchased from RiboBio (Guangzhou, China) company was incubated with the cell lysate at 37°C for 4 h, and then magnetic beads were added to separate the probe. Finally, RNA fragments were extracted, purified and quantified by qPCR.

ChIP

A SimpleChIP Plus Enzymatic Chromatin IP Kit (Cell Signaling Technology, USA) was used to carry out the experiment according to the manufacturer's directions. We first added formaldehyde to the cell culture dishes in order to crosslink the protein with DNA. After that, glycine was added to terminate the crosslinking and the cells were scraped off the plate. Then, the DNA was digested to a length of about 150-900bp, and the nucleus was completely cleaved using an ultrasonic crusher. Antibodies were added to the lysate at 4 °C overnight. The antibodies used in this paper include Normal Rabbit IgG (Cell Signaling Technology, #2729), Histone H3 Rabbit mAb (Cell Signaling Technology, #4620) and anti-beta-catenin (Abcam, ab32572). ChIP-Grade Protein G Magnetic Beads (Cell Signaling Technology, #9006) were added to the sample and incubated at 4 °C for 2 hours. The chromatin was eluted by gently swirling mixing and incubating at 65 °C for 30 min followed by adding NaCl and proteinase K at 65 °C for 2h. Finally, DNA was purified and detected by qPCR.

Luciferase reporter assays.

For luciferase reporter experiments, 293T cells were digested and seeded into 6-well plates (5×10^5 cells/well). Luciferase reporter constructs were co-transfected into 293T cells with related expression plasmid by using Lipo-fectamine 3000 Reagent (Thermo Fisher Scientific, USA). After 48 hours, the cells were lysed by adding appropriate amount of reporter gene lysate. Then, Firefly luciferase and Renilla luciferase were added to detect the luciferase activities, respectively. The Dual Luciferase Reporter Assay kit (GeneCopoeia, China) was used to detect the luciferase activity according to the manufacturer's directions.

TCGA and RNA-seq analysis

Gene expression data of normal colorectal tissues and colorectal tumor samples were downloaded from the Genomic Data Commons (GDC, <https://portal.gdc.cancer.gov>). RNA-seq was performed by Illumina HiSeq 2000 to screen out differentially expressed genes. Differentially expressed genes were defined when the fold change ≥ 1 and adjusted P value < 0.05 between tumor and normal samples.

Statistical analysis

GraphPad Prism version 7.0 was used for mapping and statistical analysis in this study. And unpaired / paired Student's t test, ANOVA or Log-Rank test (Kaplan-Meier curves) were used to determine statistical significance. The sample size and the number of experimental repetitions were noted in figure legends.

Results

Identifying a specific up-regulated lncRNA in CRC

In the previous study by us, we have determined the temporal expression profile of lncRNAs in CRC initiation^[18]. A comparison of lncRNAs expression profile in colonic tissues between normal mice and AOM/DSS-induced colitis-associated cancer model indicated that lncRNA-mAK028845 was significantly

up-regulated during CRC initiation (Figure 1A). Further results suggested that lncRNA-mAK028845 was widely expressed in normal tissues of mice (Figure 1B). Sequence homology alignment indicated that Homo sapiens lncRNA-AK028845 (named as specific up-regulated lncRNA in CRC, SURC) was located on chromosome 17 and contained the exon of KRT37 gene and the pseudogene KRT41 (Supplementary Figure 1). Furthermore, we screened the differentially expressed genes between colon cancer and adjacent tumors in TCGA database to clarify the similarity expression profile of SURC between human and mice. The volcano map indicated that SURC was significant upregulated in malignant tissues of colorectal cancer patients, compared with adjacent normal colorectal tissues (Figure 1C). In order to determine the distribution and expression of SURC, FISH-staining was employed to detect SURC in normal tissues microarrays and cancer tissues microarrays containing 13 kinds of solid tumors (Figure 1D). The results demonstrated that the expression of SURC was relatively high in stomach, duodenum, liver, pancreas, lung, seminal vesicle, cerebellum and brainstem. And SURC was moderate expressed in thyroid, esophageal, jejunum, ileum, appendix, colon, rectum, trachea, prostate and medulla oblongata, whereas in other tissues such as spleen, telencephalon and muscle, the expression of SURC was low (Figure 1E). Further analysis demonstrated that SURC was highly expressed in a variety of tumors including gastric cancer, lung cancer and CRC (Figure 1F), but SURC was up-regulated only in colon cancer tissues and rectal cancer tissues compared with adjacent normal tissues (Figure 1F). These results suggested that SURC was a specific up-regulated lncRNA in CRC.

SURC correlates with poor prognosis of CRC patients

To investigate the expression and potential prognosis prediction role of SURC in CRC, qPCR and FISH staining were employed to determine SURC expression in 150 surgical samples of CRC patients. qPCR results indicated that the expression of SURC in malignant tissues was significantly higher than that in adjacent normal colorectal tissues (Figure 2A). More SURC positive cells were detected by FISH staining in CRC tissues, compared with adjacent normal colorectal tissues (Figure 2B). The analysis of CRC samples in The Cancer Genome Atlas (TCGA) database also confirmed that the significant up-regulation of SURC in malignant tissues of CRC, compared with normal colorectal tissues (Figure 2C). qPCR analysis also suggested that SURC expression was significant up-regulated in most CRC cell lines such as SW480, HCT116, LoVo and SW620 cells, compared with normal intestinal epithelial cells (Supplementary Figure 2). Further analysis based on FISH staining in HXCRC cohort (containing 140 pairs of CRC tissues) indicated that SURC was significantly up-regulated in stage II / III patients than that in stage I patients (Figure 2D). In addition, we demonstrated that higher SURC expression was negatively correlated with poorer disease-free survival and overall survival in CRC (Figure 2E&2F). Similarly, the analysis in TCGA-COAD database also showed that CRC patients with high SURC expression associated with poorer disease-free survival and overall survival (Figure 2G&2H). Collectively, SURC was up-regulated in CRC tissues and correlated with poor prognosis of CRC patients.

APC mutation promoted SURC expression in CRC

To investigate the molecular mechanism under specific up-regulation of SURC in CRC, we first analyzed the expression correlation between SURC and KRT37, which contained a length of similar sequence. The results indicated that there was a positive correlation between them in CRC samples (Supplementary Figure 3A). However, overexpression of KRT37 in LoVo and SW620 cells did not affect the expression of SURC (Supplementary Figure 3B). Meanwhile, knockdown of SURC did not significantly regulate the expression of KRT37 (Supplementary Figure 3C). These results indicated that there was no expression regulation between SURC and KRT37.

Next, we analyzed the correlation between SURC expression and most frequently mutated genes in CRC based on TCGA-COAD database and demonstrated that the expression of SURC was significantly up-regulated in APC mutated CRC samples compared with APC wild type samples (Figure 3A, Supplementary Figure 3D). Higher expression of murine SURC was also detected in colorectal tissues of APC^{min/-} mice, compared with APC^{WT} mice (Figure 3B). Ectopic expression of wild type APC in SW620 and LoVo cells significantly inhibit SURC expression (Figure 3C). Meanwhile, knockdown of APC in human colon epithelia cell dramatically promoted SURC expression (Figure 3D). APC is a negative regulatory factor of β -catenin, which functions as a transcription factor. Thus, we speculated that APC mutation led to the increased expression of SURC via promoting β -catenin expression in CRC. To clarify if β -catenin had regulatory effects on SURC expression, we treated CRC cells with PNU-75654 (an inhibitor of β -catenin) and SKL2001 (an activator of β -catenin), respectively. We found that the decreased expression of β -catenin could inhibit the expression of SURC, while the activated β -catenin protein level could promote SURC expression (Supplementary Figure 3E&F). Furthermore, activation of β -catenin efficiently attenuated APC-mediated downregulation of SURC in SW620 cells (Figure 3E&F), while inhibition of β -catenin efficiently blocked APC knockdown induced SURC upregulation in human colon epithelia cell (Figure 3G&H). Further informatic analysis based on PROMO database (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) predicted that β -catenin could bind to SURC promoter sites, and ChIP results confirmed the binding efficiency of β -catenin on the promoters of SURC (Figure 3I). These results indicated that APC mutation increased the expression of SURC by promoting the binding of β -catenin with SURC promoter.

Knockdown of SURC inhibits CRC growth

To clarify the regulatory effect of SURC on the growth of CRC, we stably knocked down SURC in SW620 and LoVo cells through lentivirus mediated shRNA targeting SURC (Fig. 4A). Then, the cells were injected into mice for subcutaneous tumor model establishment, and the results confirmed the decreasing of SURC in SURC knockdown tumor tissues (Figure 4B, Supplementary Figure 4A&4B). Our results suggested that knockdown of SURC significantly inhibits LoVo tumor growth by 60.7% (shSURC-575) and 67.3% (shSURC-1083) in tumor volume (Figure 4C) and by 53.8% (shSURC-575) and 66.7% (shSURC-1083) in tumor weight (Figure 4D). Similar inhibition of tumor volume and tumor weight were also observed in SW620 tumors (Figure 4E&4F). In vitro results indicated that silencing SURC expression effectively inhibit CRC cell proliferation (Figure 4G) and colony formation (Figure 4H). After that, we obtained RKO cells which stably overexpressed SURC to further determine the function of SURC. Then, we

detected up-regulation of SURC in RKO cells (Figure 4I) and subcutaneous tumors (Figure 4J, Supplementary Figure 4C) by qPCR and FISH staining. Consistent with the previous results, we found that overexpression of SURC significantly promotes the growth of subcutaneous tumor (Figure 4K&4L), increases cell proliferation (Figure 4M) and colony formation of RKO cells (Figure 4N). These results indicated that SURC functions as an oncogene in CRC.

SURC directly binds miR-185-5p

To determine whether SURC promoted the growth of CRC depends on the presence of miRNA, we transfected SW620 and LoVo cells with siRNA targeting Dicer (Supplementary Figure 5A). Cell proliferation assay and colony formation assay indicated that blocking miRNA synthesis by knockdown of Dicer efficiently attenuating the regulation of SURC on the growth and colony formation of CRC cells (Figure 5A&5B). ChIRP results also showed that few proteins bound to SURC (Supplementary Figure 5B). These results suggested that SURC promoted the growth of CRC depending on miRNA.

In order to find the target miRNA interacting with SURC, we detected the miRNA expression profiles in control and SURC knockdown cells. The results suggested that 11 miRNAs were down-regulated and 3 miRNAs (miR-185-5p, miR-220c and miR-378a-3p) were up-regulated in knockdown SURC cells compared with control (Figure 5C). Furthermore, ChIRP experiments showed that SURC directly targeted with miR-185-5p rather than miR-220c and miR-378a-3p (Figure 5D). FISH staining results indicated that SURC was mainly located in the cytoplasm of SW620 and LoVo cells (Supplementary Figure 5C), which was further confirmed by qPCR results based on nuclear cytoplasmic separation (Supplementary Figure 5D). As shown in Figure 5E, we demonstrated that SURC and 185-5p co-localized in cytoplasmic of CRC cells by FISH staining (Figure 5E). Target prediction suggested that miR-185-5p directly binds 2311-2333 region of SURC, which contains 14 binding sites (Figure 5F). Dual-luciferase reporter results shown that miR-185-5p inhibit the transcription activity of SURC (Supplementary Figure 5E). Then, SURC was divided into five truncated vectors based on the predicted binding sites of miR-185-5p, and the results suggested that SURC (2085-2521 region) was the binding region of miR-185-5p by dual-luciferase reporter assay (Figure 5G). The transcription activity of SURC with mutated binding sites of miR-185-5p was not affect by miR-185-5p in CRC cells (Figure 5H). Next, we constructed lentivirus mediated wild type and mutated overexpression plasmids of 2085-2521 region and were used to infect RKO cells. The results showed that overexpression of wild type SURC²⁰⁸⁵⁻²⁵²¹ significantly promote cell proliferation, colony formation and subcutaneous tumor growth, while overexpression of the mutant SURC²⁰⁸⁵⁻²⁵²¹ rescue this increase (Figure 5I-5K, Supplementary Figure 5F). These results suggested that miR-185-5p was a direct target for SURC to promote the growth of CRC.

SURC inhibits miR-185-5p expression in CRC

Based on the above findings, we intended to further clarify the regulatory effect of SURC on miR-185-5p expression. We demonstrated that knockdown of SURC significantly up-regulated miR-185-5p expression in CRC cells and subcutaneous tumors (Figure 6A&6B, Supplementary Figure 6A), while ectopic

expression of SURC dramatically inhibited miR-185-5p expression in RKO cells and subcutaneous tumors (Supplementary Figure 6B&6C). miR-185-5p expression was also determined in 75 malignant tissues of CRC patients and low miR-185-5p expression was found in SURC high expression tissues (Figure 6C). Further analysis demonstrated the negative correlation between SURC expression and miR-185-5p expression in malignant tissues of CRC patients (Figure 6C). Based on the miR-185-5p determination in CRC tissues, we demonstrated that miR-185-5p expression was significantly decreased in CRC tissues compared with normal tissues (Figure 6D) and miR-185-5p expression was positively correlated with disease-free survival and overall survival of CRC patients (Figure 6E&6F). In order to clarify the potential necessary role of miR-185-5p under SURC regulating growth of CRC, we treated the cells with miR-185-5p inhibitor (Supplementary Figure 6D) and demonstrated that miR-185-5p inhibitor efficiently rescue the regulation role of SURC on CRC cell proliferation and colony formation (Figure 6G&6H, Supplementary Figure 6E). These results indicated that miR-185-5p plays a necessary role during SURC promoting CRC growth.

To further investigated the mechanism under SURC inhibiting miR-185-5p expression, we detected the expression of pri-miR-185-5p. Our results suggested that the expression of pri-miR-185-5p was significantly decreased in SURC knockdown CRC cells (Figure 6I). Then the effect of SURC on miR-185-5p degradation was investigated by adding RNA synthesis inhibitor actinomycin d. Our results suggested that knockdown of SURC inhibited the degradation of miR-185-5p in CRC cells (Figure 6J), while knockdown of SURC promoted the synthesis of miR-185-5p from pri-miR-185-5p (Figure 6K). Collectively, SURC inhibited the synthesis of miR-185-5p from pri-miR-185-5p via binding with miR-185-5p, which resulted in miR-185-5p down-regulation in CRC cells.

SURC regulates the activity of miR-185-5p/CCND2 axis

To further elucidate the downstream targets of SURC in regulating CRC growth by targeting miR-185-5p, we detected the mRNA expression profiles in SURC knockdown and control cells, and found that SURC knockdown decreases the expression of a series of coding genes that were involved in p53 signaling pathway, PI3K-Akt signaling pathway (Supplementary Figure 7A&7B). CCND2 is one of the significant down-regulated genes (Figure 7A), and the decrease of miR-185-5p was confirmed by qPCR in SURC knockdown CRC cells (Figure 7B) and subcutaneous tumor tissues (Figure 7C). Western blotting results suggested that knockdown of SURC inhibits CCND2 expression in CRC cells (Figure 7D). And less CCND2 positive cells were observed in SW620-shSURC tumors (Figure 7E). Luciferase report assay indicated that miR-185-5p inhibits the transcription of CCND2 (Figure 7F), while SURC efficiently blocks the inhibitory effect of miR-185-5p on CCND2 transcription (Figure 7G). Then the cell cycle and proliferative ability of CRC cells were investigated. As shown in Figure 7H, cell cycle analysis indicated that knockdown of SURC prevents CRC cells from transforming from G1 phase to S phase. BrdU staining indicated that knockdown of SURC inhibits the proliferative activity of CRC cells (Figure 7I). And less ki67 positive cells were also detected in SW620-shSURC tumors (Figure 7J). These studies indicated that SURC promotes CRC growth via regulating the activity of miR-185-5p/CCND2 and cell proliferation.

Discussion

In the present study, we identified a novel lncRNA (SURC) which is specific up-regulated in CRC and function as an oncogene. Mutated APC protein resulted in stabilization of β -catenin in CRC, which promotes the transcription of SURC via binding to its promoter (Figure 8, right), while wild-type APC protein caused the degradation of β -catenin, which inhibits the transcription of SURC (Figure 8, left). After transcription, SURC was transferred to cytoplasm and inhibits miR-185-5p expression via binding to miR-185-5p, which results in CCND2 expression, cell proliferation and tumor growth (Figure 8). High expression of SURC was demonstrated in malignant tissues of CRC patients and SURC expression was correlated with poorer disease-free survival and overall survival.

Previous studies have found that many lncRNAs such as CCAT2^[19, 20] and LncGata6^[21] are significant up-regulated in CRC compared with normal colorectal tissues. In the present study, we demonstrated that SURC was widely expressed in various solid tumor tissues. But further analysis suggested that SURC was only significant up-regulated in CRC, but not in other solid tumor tissues, when compared with paired adjacent normal tissues. These findings indicated that SURC expression would be an efficient diagnosis target for CRC. But further investigations based on large samples should be performed to determine the possibility, especially for the SURC expression in blood. Meanwhile, our study also indicated that high expression of SURC in tumor tissues was correlated with poorer disease-free survival and overall survival, which suggested that SURC is a predictor for prognosis of CRC patients.

APC gene is a tumor suppressor gene and plays an important role in cell proliferation, migration, adhesion, differentiation and chromosome aggregation in CRC^[22-24]. The study by Wang F et al showed that wild type APC activated lncRNA (lncRNA-APC1) was down-regulated in CRC compared with normal colorectal tissues and inhibited cell growth, angiogenesis and metastasis^[16]. However, APC mutation but not APC down-expression was found in most sporadic CRC^[25, 26], and APC mutation was one of the most important inducer for CRC tumorigenesis and progression^[27]. In the present study, we demonstrated that mutated APC protein mediated stabilization of β -catenin protein promotes the transcription of SURC via binding to its promoter. Thus, due to the high mutation frequency (78-80%) of APC in CRC and low mutation frequency of APC in other solid cancers^[28-31], specific up-regulation of SURC was demonstrated only in CRC, but not in other solid tumor tissues. The high expression of SURC in other solid tumor tissues and paired normal tissues may contribute to the base expression of β -catenin protein.

In recent years, an increasing number of studies showed that lncRNAs played a key role in regulating the biological process of CRC. The altered expression of lncRNA-NEAT1 lead to the changes of cell proliferation, invasion and migration in vivo and in vitro experiments^[32]. lncRNA-SNHG15 regulated the expression of downstream genes including MYC, NRAS, BAG3 and ERBB3 which related closely to cancer progression^[33, 34]. As a new definition lncRNA, SURC function was unclear. In the present study, we demonstrated that knockdown of SURC impaired CRC tumor growth in mice and CRC cell proliferation in vitro. Ectopic expression of SURC promoted the growth of subcutaneous transplanted tumor in mice. These novel findings clarified the function of SURC and provided a potential therapy target for CRC.

LncRNAs regulated the tumorigenesis and progression of tumor through a variety of mechanisms, including acting as microRNA sponges, modulating post-transcription, regulating the expression of proximal genes and distal genes in the nucleus^[35-37]. In the present study, our results suggested that promotion of CRC growth by SURC was miRNA dependent and few proteins were found to be bound to SURC. Based on miRNA expression sequence, ChIRP and FISH staining, we demonstrated that SURC directly interact with miR-185-5p, which was functioned as a tumor suppressor in various cancers^[38-40]. We demonstrated that miR-185-5p expression was down-regulated in SURC expressed CRC cells, which may contribute to the inhibition of synthesis of miR-185-5p from pri-miR-185-5p via the direct interaction between SURC and miR-185-5p. Further experiments with miR-185-5p inhibitor also confirmed the necessary role of miR-185-5p during SURC promoting CRC growth. Several coding genes were demonstrated as the direct targets of miR-185-5p^[41-43], and the results of sequencing and deregulated gene analysis indicated that CCND2 was inhibited in SURC knockdown CRC cells. Ectopic expression of SURC efficiently blocked miR-185-5p mediated inhibition of CCND2 transcription, which suggested that SURC promotes CRC growth via regulating the activity of miR-185-5p/CCND2 and cell proliferation. These novel findings provide solid evidences for understanding the mechanism under SURC regulating CRC growth.

Conclusion

In summary, the present study identified a novel lncRNA (SURC) that was activated by mutated APC protein and specific up-regulated in CRC tissues. High expression of SURC in tumor tissues was correlated with poorer disease-free survival and overall survival. SURC promoted CRC tumor growth via interacting with miR-185-5p and regulating the activity of miR-185-5p/CCND2 axis. These findings provided a novel diagnosis, and prognosis prediction target for CRC.

Abbreviations

APC: Adenomatous polyposis coli; CRC: Colorectal cancer; lncRNA: Long non-coding RNA; ncRNA: Non-coding RNA; EMT: Epithelial-to-mesenchymal transition; WGCNA: Weighted correlation network analysis; TCGA: The Cancer Genome Atlas; ChIP: Chromatin immunoprecipitation; CHIRP: Chromatin Isolation by RNA Purification.

Declarations

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Authors' contributions

Junshu Li, Yanhong Ji, Na Chen were involved in acquisition of the data with the assistance from Huiling Wang, Zhexu Dong, Ruiyi Jiang, iamei Fu and Xiaolan Su. Hongxin Deng and Lei Dai was involved in the study concept and design, and obtained funding. Chao Fang, Xiaonan Yin and Zhiyuan Jiang were involved in human colon cancer tissues collected. Chao Fang were involved in the prognosis analysis. Lin Cheng and Gang Shi were involved in analysis and interpretation of the data. Junshu Li, Lei Dai and Hongxin Deng were involved in drafting of the manuscript and critical revision of the manuscript for important intellectual content.

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Availability of data and materials

All data, models or code generated or used during the study are available from the corresponding author by request.

Ethics approval and consent to participate

Experimental protocols for human tissues and animal were approved by West China Hospital of Sichuan University Biomedical Research Ethics Committee (Number: 2018(280)).

Consent for publication

Not applicable.

Competing interests

The authors report no conflicts of interest in this work.

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Figures

Figure 1

Identification of SURC as a specific up-regulated lncRNA in CRC. (A) Heatmap for differentially expressed lncRNAs in AOM/DSS-induced colitis-associated cancer tissues compared with normal tissues in mice. (B) qPCR shows the expression of SURC in different tissues and organs of mice (n=3). (C) The volcano plots show the fold changes and FDR values of 1,174 lncRNA candidates in colon tumors (n=469) versus adjacent normal tissues (n=41) from the TCGA-COAD database. (D) FISH staining showing the

distribution and expression of SURC in malignant and adjacent normal tissues among different organs. Scale bars represents 100 μ m. (E) SURC levels in various normal tissues by FISH assay. (F) SURC levels in malignant and adjacent normal tissues among digestive system (**, $P<0.01$).

Figure 2

SURC up-regulated in colorectal cancer and negatively correlated with prognosis. (A) Up-regulation of SURC in malignant colorectal tissues compared with normal tissues by qPCR, n=60. (B) Higher expression of SURC was detected in CRC samples than in matched normal tissues from HXCRC cohort (n=90), which measured by FISH. Analysis of FISH score of SURC in tissues. (C) Higher expression of SURC in CRC tissues (n=469) than in adjacent normal tissues (n=41) from the TCGA database. (D) Up-regulation of SURC in II / III stage compared with stage I CRC tissues from HXCRC cohort. (E) Kaplan-Meier analysis of overall survival and (F) disease-free survival curves for CRC samples from our platform (n=140) with SURC-low (n = 70) or SURC-high (n = 70) expression (log-rank test). (G) Kaplan-Meier plots of overall survival and (H) disease-free survival curves for CRC patients from the TCGA database (n=404), higher SURC expression with poorer prognosis.

Figure 3

APC mutation promotes up-regulation of SURC in CRC. (A) Analysis of SURC expression in the mutated APC tissues compared with wild type (WT) tissues in TCGA database. (B) Detection of SURC expression in colorectal tissues of APC^{WT} and APC^{min/-} mice (n=3, **, P < 0.01). (C) SURC expression was detected by qPCR in APC-overexpressing cells compared with control cells. (n=3, **, P < 0.01). (D) SURC expression was detected by qPCR in HCoEpiC cells infected with lenti-shNC and lenti-shSURC (n=3, **, P < 0.01). (E) Western blotting shows the expression of β-catenin in SW620-Ctrl and SW620-APC cells treated with SKL2001 and DMSO. GAPDH was used as a loading control. (F) qPCR shows the expression of SURC in SW620-Ctrl and SW620-APC cells treated with SKL2001 and DMSO (n=3, **, P < 0.01). (G) Western blotting shows the expression of β-catenin in HCoEpiC-shNC and HCoEpiC-shAPC cells treated with PNU-74654 and DMSO. GAPDH was used as a loading control. (H) qPCR shows the expression of SURC in HCoEpiC-shNC and HCoEpiC-shAPC cells treated with PNU-74654 and DMSO (n=3, **, P < 0.01). (I) qPCR analyze the expression of SURC promoter in products by ChIP (chromatin immunoprecipitation) assay (n=3, **, P < 0.01).

Figure 4

SURC promotes cell proliferation and tumor growth in vivo and in vitro. (A) SW620 and LoVo cells were transfected with shNC or shSURC and detected by qPCR ($n=3$, **, $P < 0.01$). (B) Detection of SURC expression in subcutaneous tumors by FISH (Scale bar represents 100 μ m). Analysis of SURC positive cells in each frame ($n=3$, **, $P < 0.01$). (C-F) Tumor volume and tumor weight were measured in SW620 and LoVo tumors and compared among groups ($n=5$, **, $P < 0.01$). (G) CCK8 assay shows relative cell

growth at 0, 24, 48, 72h (n=5, **, P < 0.01). (H) The colony formation pictures of SW620 and LoVo cells infected with shNC and shSURC. Three biologically independent experiments were performed (n=3, **, P < 0.01). (I) RKO cells were infected with lenti-SURC and detected by qPCR (n=3, **, P < 0.01). (J) FISH staining shows SURC expression of xenograft tumors from mice subcutaneously injected with control or SURC-overexpressing RKO cells (Scale bar represents 100 μ m). Analysis of SURC positive cells in each frame (n=3, **, P < 0.01). (K-L) Tumor volume and tumor weight were measured in RKO tumors among various groups (n=5, **, P < 0.01). (M) CCK8 assay shows relative cell proliferation at 0, 24, 48, 72h in RKO cells (n=5, **, P < 0.01). (N) Effects of SURC overexpression on colony formation in RKO cells (n=3, **, P < 0.01).

Figure 5

Direct binding of SURC to miR-185-5p. (A) CCK8 assay shows the relative cell growth in SW620 cells transfected with siNC or siDicer ($n=5$, **, $P < 0.01$; ns, no significant difference). (B) Colony formation of SW620 cells transfected with siNC or siDicer ($n=3$, **, $P < 0.01$). (C) Altered microRNA expression in shSURC or shNC-treated colorectal cancer cells by RNA sequencing. (D) ChIRP assay detected the ability of SURC direct binding to miRNA in SW620 cells ($n=3$, **, $P < 0.01$). (E) FISH assay shows the co-

localization of SURC and miR-185-5p, Scale bar represents 10 μm . (F) Prediction of binding sites of SURC and miR-185-5p by bioinformatics analysis. (G) The dual-luciferase reporter system confirmed 2085-2521 segment of SURC was combined with miR-185-5p ($n=3$, **, $P < 0.01$). (H) The dual-luciferase reporter system shows the combination between miR-185-5p and SURC core region by transfected with SURC2085-2521WT or MUT plasmid ($n=3$, **, $P < 0.01$). (I) Colony formation shows the function of SURC core region in RKO cells which was transfected with SURC2085-2521WT or MUT plasmid ($n=3$, **, $P < 0.01$). (J) Tumor volume and (K) tumor weight were measured in RKO cells infected with SURC2085-2521 MUT compared with WT cells ($n=5$, **, $P < 0.01$).

Figure 6

SURC modulated colorectal cancer progression by regulating the expression of miR-185-5p. (A) qPCR shows the expression of miR-185-5p in LoVo and SW620 cells (n=3, **, P < 0.01). (B) FISH assay shows the expression of miR-185-5p in subcutaneous tumor tissue of SW620 cells (Scale bar represents 100 μ m). Analysis of miR-185-5p positive cells in each frame (n=3, **, P < 0.01). (C) Correlations between the SURC levels and the miR-185-5p levels in CRC patients of HXCRC cohort (n=75) were determined by FISH

staining (Scale bar represents 200 μm). The r values and P values are from Pearson's correlation analysis. (D) Lower expression of miR-185-5p was detected in CRC samples than in matched normal tissues from HXCRC cohort ($n=90$), which was measured by FISH. (E) Kaplan-Meier plots of overall survival and (F) disease-free survival for CRC samples from HXCRC cohort ($n=140$). (G) Cell viability and (H) colony formation was examined after administration with miR-185-5p inhibitor ($n=5$, **, $P < 0.01$). (I) qPCR shows the expression of pri-miR-185-5p in LoVo and SW620 cells infected with lenti-shNC and lenti-shSURC ($n=3$, **, $P < 0.01$). (J) Levels of miR-185-5p and (K) pri-miR-185-5p were examined at different times after administration with actinomycin D ($n=3$, **, $P < 0.01$).

Figure 7

SURC regulated CRC cell cycle by affecting CCND2 expression. (A) SURC-regulated gene expression by RNA-seq. (B) qPCR shows the expression of CCND2 in LoVo and SW620 cells infected with lenti-shNC and lenti-shSURC (n=3, **, P < 0.01). (C) qPCR shows the expression of CCND2 in subcutaneous tumors of LoVo and SW620 cells (n=3, **, P < 0.01). (D) Western blotting shows the expression of CCND2 in LoVo and SW620 cells. GAPDH was used as a loading control. (E) Immunohistochemistry (IHC) staining of

CCND2 in the subcutaneous tumors of shNC and shSURC cells. Analysis of CCND2 positive cells (n=3, **, P < 0.01). (F) Dual-luciferase reporter assays to test the interaction between miR-185-5p and CCND2 by using a synthetic miR-185-5p mimic co-transfected with CCND2-wt or CCND2-mt constructs (n=3, **, P < 0.01). (G) Dual luciferase assay of SW620 cells co-transfected with the CCND2 reporter constructs (wild-type or mutants), the SURC overexpressing plasmids and miR-185-5p mimic (n=3, **, P < 0.01). (H) The cell cycle was analyzed by flow cytometry analysis in SW620 cells (n=3, **, P < 0.01). (I) BrdU assays of the SW620 cells with SURC knockdown by shRNAs compared with the control. (Scale bar represents 100 μ m, n=3, **, P < 0.01). (J) Immunohistochemistry (IHC) staining of Ki67 in the shNC and shSURC subcutaneous tumors (Scale bar represents 100 μ m). Analysis of Ki67 positive cells in each frame (n=3, **, P < 0.01).

Figure 8

Molecular mechanism model for SURC in CRC cells. Mutated APC protein resulted in stabilization of β -catenin in CRC, which promotes the transcription of SURC via binding to its promoter (right), while wild-type APC protein caused the degradation of β -catenin, which inhibits the transcription of SURC (left). After transcription, SURC was transferred to cytoplasm and inhibits miR-185-5p expression via binding to miR-185-5p, which results in CCND2 expression, cell proliferation and tumor growth.

Supplementary Files

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