LncRNA SNHG6 inhibits cell proliferation and metastasis by targeting ETS1 via the PI3K/AKT/mTOR pathway in colorectal cancer

SU MENG, ZHAO JIAN, XIAOFEI YAN, JIBIN LI and RUI ZHANG

Department of Medical Oncology, Cancer Hospital of China Medical University, Liaoning Cancer Hospital and Institute, Shenyang, Liaoning 110042, P.R. China

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Abstract. The term ‘long non-coding RNAs’ (lncRNAs) refers to all non-protein coding transcripts >200 nucleotides in length. Dysregulation of lncRNAs has been identified in colorectal cancer, which is one of the more serious types of cancer worldwide, third place in the mortality rates, and is associated with poor prognoses. Novel evidence suggests that lncRNAs serve an important role in regulating the development and progression of colorectal cancer. In the present study, it was demonstrated that SNHG6 expression was downregulated in colorectal cancer tissues by reverse transcription quantitative polymerase chain reaction assays; however, ETS1 expression levels were upregulated. Overexpression of SNHG6 not only inhibited the proliferation of colon cancer cells in vitro by inducing apoptosis, but also inhibited cell proliferation, invasion and migration. The overexpression of SNHG6 inhibited colon cell viability and proliferation by targeting ETS1 through the phosphoinositide 3-kinase/protein kinase B/mechanistic target of rapamycin signaling pathway. These results suggested that SNHG6 may directly suppress ETS1, which may be one of potential mechanisms through which it inhibits the viability and proliferation of colorectal cancer cells, and it provides novel insight into the carcinogenesis of colorectal cancer. In addition, it may assist in the development of a treatment approach for ETS1-activated colorectal cancer.

Introduction

The incidence of colorectal cancer is increasing globally. A total of ~655,000 people succumb to colorectal cancer each year. The 5-year survival rate of early colorectal cancer is ~90%, but this rate decreases to 15% in metastatic colorectal cancer (1). Although numerous protocols for early screening and treatment have been developed in previous years, there has been no significant increase in the survival rate of colorectal cancer in the past 20 years (2). In addition, the most common cause of treatment failure is metastasis. This process is multifaceted, involving cancer cell invasion, epithelial mesenchymal transition (EMT), extracellular matrix degradation, angiogenesis and microenvironment chemotactic action. The abnormal activation or inactivation of a number of oncogenes or tumor suppressor genes and signaling pathways are regulated at transcriptional, post-transcriptional and translational levels, and the regulatory mechanisms are complex (3).

Colorectal cancer is one of the three most common fatal types of tumor, with up to 1.03 million cases each year and 530,000 mortalities each year in developed countries including Western Europe and the United States of America. According to China's Ministry of Health, the incidence of colorectal cancer is the third-highest incidence of all cancers (4). Colorectal cancer is a malignant tumor caused by multiple factors, including individual genetics and environmental effects. For example, large-scale genetic studies have identified that the occurrence of colorectal cancer is associated with multiple genes abnormal expression and multiple molecular interactions (5-7), but the studies investigating the molecular mechanism of colorectal cancer incidence are not exhaustive. Therefore, additional studies exploring the pathogenesis of colorectal cancer may provide the theoretical basis for novel clinical anti-tumor drugs (8).

Long non-codingRNAs (lncRNA) are a type of molecule within the transcriptome in the cell nucleus or cytoplasm, similar to mRNA, with a length >200 nucleotides. Previous studies have demonstrated that lncRNA is closely associated with the occurrence of cancer (9,10). Abnormal lncRNAs may serve key roles as tumor suppressor genes or oncogenes. Colorectal cancer has one of the most rapidly increasing morbidity rates and has become a threat to health and life. (11).
Studies investigating this association between lncRNAs and cancer have indicated that non-small cell lung cancer genome-wide microarray data that small nucleolar RNA host gene 6 (SNHG6), LINC00649, DLX6-AS1 and 3 lncRNA were identified to be abnormally expressed in patients with lung cancer (9-11), but no study has demonstrated whether lncRNA SNHG6 serves a significant role in colorectal cancer.

The normal human E26 transformation specific-1 (ETS1) gene is a proto-oncogene. It has been suggested that the ETS1 gene is involved in cell growth and extracellular matrix invasion, which promotes tumor invasion and metastasis (12). Previous studies have indicated that the expression of ETS1 is increased in a number of malignant tumors (12,13). The expression of ETS1 and its roles in tumor invasion and metastasis have attracted widespread attention.

The present study investigated the role of lncRNA SNHG6 in the development of colorectal cancer; the results identified that the expression of lncRNA SNHG6 was inhibited in tumor cells and tissues, and that the overexpression of lncRNA SNHG6 in cells may suppress ETS1 expression, inhibit cell proliferation. In addition, it was confirmed that the lncRNA SNHG6 inhibited ETS1 expression by directly targeting its 3'-untranslated regions (UTR) and activated the intrinsic invasion pathway through downregulating expression of phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)/mechanistic target of rapamycin (mTOR).

Materials and methods

Colorectal cancer tissue samples and the variant choice of SNHG6. A total of 30 colon tumors and adjacent non-tumor tissues samples were collected from patients with colorectal cancer from the Cancer Hospital of China Medical University (Shenyang, China) between April and September 2017. There were 18 males and 12 females; the age range was 34-82 years old, with a median age of 52 years. Patients were diagnosed according to the World Health Organization colon cancer histology classification and grading standards 2010 (14). The colon cancer staging system proposed by the American Cancer Association (AJCC) has become widely recognized as an independent indicator that can comprehensively reflect the progress of malignant tumors and judge prognosis (15). All patients with colon tumors enrolled in the present study did not receive any treatment prior to surgery. This was due to the fact that different radiotherapy or chemotherapy regimens may affect the outcomes of genetic studies of colon cancer, and it is difficult to standardize the frequency and intensity of different chemotherapy and radiotherapy treatments within the patient cohort. The protocol was approved by the Ethics Committee of Cancer Hospital of China Medical University, and written informed consent was obtained from all study participants. SNHG6 has several variants; from previous optimization experiments (16) it was identified that only variant 1 successfully completed the editing of the gene, so variant 1 was used to establish the overexpression model. Concomitantly, subsequent experiments within the present study confirmed that this variant was effective.

Cell culture and transfection. The human colon cancer SW480 and HCT-116 cell lines and normal colonic mucosa NCM460 cell line were purchased from American Type Culture Collection (Manassas, VA, USA). These cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and incubated in a humidified atmosphere containing 5% CO2 at 37°C. LncRNA pWPXL-SNHG6 and small interfering (si)-SNHG6 and si-negative control were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China).

For transfection, cells at a density of 1x10^5 cells/well were seeded in each well of a 24-well microplate, grown for 24 h to reach 30-50% confluence and then incubated with 50 nM LncRNA pWPXL-SNHG6 and small interfering si-SNHG6 and si-negative control with Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) in 100 µl serum-free DMEM, according to the manufacturer’s protocols.

RNA isolation and reverse transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from SW480 and HCT-116 cell lines using TRIzol® (Thermo Fisher Scientific, Inc.). The corresponding RNA was reverse transcribed into cDNA using a QuantiFast SYBR Green PCR kit (Qiagen GmbH, Hilden, Germany). Then, RT-qPCR analysis was detected using the SYBR Green qPCR Master Mix (5 µl; Invitrogen; Thermo Fisher Scientific, Inc.) on a 7500 Fast system (Applied Biosystems; Thermo Fisher Scientific, Inc.). For the PCR experiments, the following forward and reverse primers were used: SNHG6; Forward, 5'-TGc caG caG TGa CTa G-3'; and reverse, 5'-TACGGAGGTGAGTGC CCAT-3'; and reference gene GAPDH; Forward, 5'-CAAGG TGATCACAGTTCAAG-3' and reverse, 5'-GTGAGCCAT TATCACCAGA-3'.

The PCR conditions included an initial denaturation step of 94°C for 2 min, followed by 30 cycles of 94°C for 30 sec, 59°C for 30 sec, 72°C for 2 min and a final elongation step at 72°C for 10 min. Taq DNA polymerase was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). GAPDH was used as an internal control to normalize gene expression. The relative gene expression levels were calculated using the ΔΔCq method (17).

Western blot analysis. Protein samples from cells were homogenized using radioimmunoprecipitation assay lysis buffer (Invitrogen; Thermo Fisher Scientific, Inc.). The protein concentrations of the cell extracts were then measured using Bradford protein dye reagent (Bio-rad laboratories, Inc., Hercules, CA, USA). A total of 30 µg/lane protein was loaded. The proteins lysates were separated on a 10% SDS-PAGE separation gel and transferred onto a nitrocellulose membrane by 300 mA for 90 min, blocked with 5% bovine serum albumin (Gibco; Thermo Fisher Scientific, Inc.) for 2 h at room temperature, and incubated with primary antibodies (Abcam, UK): anti-ETS (1:2,000; ab26096), anti-p-PI3K (1:2,000; ab151549), anti-PI3K (1:2,000; ab32089) anti-p-AKT (1:3,000; ab8932), anti-AKT (1:2,000; ab8932), anti-p-mTOR (1:2,000; ab109268), anti-mTOR (1:2,000; ab2732) and anti-GAPDH (1:1,000; ab9485) at 4°C overnight. Then, membranes were incubated at room temperature for 2 h with anti-IgG conjugated with horseradish peroxidase secondary antibodies (1:5,000; ab97040) before being visualized using the SuperSignal West Pico Chemiluminescent Substrate Trial kit (Pierce Protein
Biology; Thermo Fisher Scientific, Inc.). Images were obtained using the ChemiDoc XRS system with Quantity One software (Bio-Rad Laboratories, Inc.). Protein expression was analyzed using BandScan 5.0 software (Glyko, Inc., Novato, CA, USA). All experiments were repeated three times.

**Cell proliferation analysis.** Cells proliferation was detected in vitro using an MTT assay. Briefly, following transfection, HCT-116 cell lines were seeded at a density of 2,000 cells/well in 96-well plates and all groups were incubated for 24, 48, 72 or 96 h. MTT was added into each well (5 mg/ml; Sigma-Aldrich; Merck KGaA), and culture was maintained at 37˚C for 4‑6 h. Samples were measured at 570 nm using the MTT cell proliferation kit (Cayman Chemical Company, Ann Arbor, MI, USA).

**Flow cytometry.** For the cell apoptosis analysis, HCT116 cells were harvested 48 h after transfection and immobilized in 70% ethanol at -20˚C for 30 min. Then, the cells were resuspended in 10 ml RNase, and stained with propidium iodide (PI; 1:200) and Annexin V-FITC (1:200; BD Pharmingen, San Diego, CA, USA) for 30 min at 37˚C. A total of 500 µl PBS was added and mixed thoroughly. The samples were then analyzed by flow cytometry (BD Biosciences). Apoptotic rate of cells was analyzed by flow cytometry (BD Biosciences) using WinMDI software (version 2.9; BD Biosciences). All experiments were repeated 3 times.

**Transwell invasion assays.** The pore size in the bottom membrane of the Transwell chambers (Corning Incorporated, Corning, NY, USA) was 8 µm. The chambers were coated with Matrigel (Sigma-Aldrich; Merck KGaA) for the determination of cell invasive abilities at room temperature for 24 h. HCT116 cells were grown to ~80% confluence in the 24-well Transwell plates. Then, after 24 h incubation at 37˚C in 15% fetal bovine serum medium, the invasion of cells was measured using light microscopy at a magnification of x200 (Shanghai Bime Instrument Co., Ltd., Shanghai, China).

**Cell scratch assay.** HCT116 cells (5x10⁴ cells/well) were seeded in a 6-well plate (5x10⁴ cells per well). Lines were then drawn behind the plate with a ruler. Following attachment of the cells, a scratch was made across the middle of the wells, the width of a 10 µl pipette tip. Non-adherent cells were removed by washing with PBS 3 times, and then serum-free medium (Gibco; Thermo Fisher Scientific, Inc.) was added. Cells were incubated at 37˚C and 5% CO₂. Images were captured at 0 and 48 h.

**Statistical analysis.** Data are presented as the mean ± standard deviation of 3 independent experiments. The results were analyzed using a Student t-test or a one-way analysis of variance with Tukey's post-hoc test. All the data were analyzed using SPSS Statistics 19.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) with Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). P<0.05 was considered to indicate a statistically significant difference.

**Results**

**LncRNA SNHG6 is downregulated in colon cancer tissues and cell lines.** A previous study demonstrated that SNHG6...
is upregulated in hepatocellular carcinoma and indicates the possibility of high recurrence in cancer patients (9). However, the expression of IncRNA SNHG6 has not been determined in colon cancer. Firstly, the expression of IncRNA SNHG6 was detected in 30 pairs of colon cancer and adjacent tissues by RT-qPCR. The RT-qPCR results verified that the expression of IncRNA SNHG6 was markedly decreased in colon cancer tissues compared with the normal tissues (Fig. 1A). Furthermore, similar results were observed when examining colon cancer cell lines, in which the expression of IncRNA SNHG6 was markedly decreased compared with the normal colon cell line (Fig. 1B). In addition, the expression of ETS1, which is a transcription factor, was detected. The western blot analysis results suggested that ETS1 was highest in colon cancer cell lines (Fig. 1C). These data indicate that IncRNA SNHG6 may serve a key role in colon cancer progression.

**LncRNA SNHG6 represses colon cancer proliferation.** To additionally explore the role of IncRNA SNHG6 in colon cancer cells, the cell proliferation rates were detected in vitro following transfection with pWPXL-SNHG6 plasmids and si-SNHG6 to overexpress or silence IncRNA SNHG6 expression, respectively. The transfection efficiency was detected by RT-qPCR. The RT-qPCR results demonstrated that IncRNA SNHG6 expression was effectively upregulated following transfection with pWPXL-SNHG6; conversely, IncRNA SNHG6 expression was markedly decreased in cells following transfection with si-SNHG6 (Fig. 2A). Furthermore, an MTT assay was used to detect the proliferation of colon cancer cells. As demonstrated in Fig. 2B, the proliferation of colon cancer cells was significantly inhibited following overexpression of IncRNA SNHG6, whereas inhibition of IncRNA SNHG6 significantly increased levels of cell growth. The western blot analysis results suggested that the levels of ETS1 were downregulated when transfected with pWPXL-SNHG6 and upregulated when transfected with si-SNHG6 (Fig. 2C). In conclusion, IncRNA SNHG6 inhibited the proliferation of colon cancer cells.

**LncRNA SNHG6 inhibits proliferation and induces apoptosis of colon cancer cells.** Flow cytometry was used to detect the expression of the Annexin V and PI. As indicated in Fig. 3A, the apoptosis levels were identified to be increased when IncRNA SNHG6 was overexpressed in colon cells, and...
decreased when lncRNA SNHG6 was inhibited in colon cells (Fig. 3a and B). Subsequently, the Transwell assays suggested that the level of invasion was decreased when lncRNA SnHG6 was overexpressed in HCT116, and was upregulated following transfection with si-SnHG6 in colon cancer cells (Fig. 3c and d).

LncRNA SNHG6 inhibits migration of colon cancer cells. Cell scratch assays were used to detect the migration of colon cancer cells. Overexpression of lncRNA SNHG6 in HCT116 cells caused a decrease in cell migration levels, while transfection with si-SnHG6 resulted in an increase in the levels of migration in colon cancer cells (Fig. 4A and B).

LncRNA SNHG6 regulates cell proliferation through the PI3K/AKT/mTOR signaling pathway. Previous data have demonstrated that the PI3K/AKT/mTOR signaling pathway promotes the development and progression of colon cancer (18,19). Therefore, the protein expression levels of phosphorylated-PI3K (p-PI3K), p-AKT, p-mTOR, PI3K, AKT and mTOR were detected by western blot analysis. The results demonstrated that the p-AKT, p-PI3K and p-mTOR expression levels were markedly decreased following overexpression of lncRNA SNHG6 (Fig. 5A and B). These results suggest that lncRNA SNHG6 regulates cell proliferation through the PI3K/AKT/mTOR signaling pathway in colon cancer.

Discussion
Colon cancer is one of the most common malignant tumors in clinical practice, and its morbidity and mortality rates are increasing in the majority of countries and regions throughout the world, which poses a serious threat to human health. The primary causes of mortality in these patients are invasion, metastasis and chemotherapy resistance. The mechanism of metastasis of colon cancer is complicated, and involves the abnormal expression of multiple oncogenes and tumor suppressor genes; previous studies have identified that abnormal lncRNA expression is also an important factor (9-11). Mature lncRNA may be located in the mRNA noncoding regions (3'-UTR or introns area) of their target gene, and may result in complete or incomplete complementary pairing, degradation...
or inhibition of mRNA translation and post-transcriptional alterations in gene expression. They may also participate in the regulation of cell differentiation, proliferation, apoptosis, and tissue and organ development (5,7,17).

Previous studies have confirmed that lncRNA may regulate the expression of at least 30% of human protein-coding genes (18), and serve a larger role compared with protein-coding genes (19,20). In a variety of human tumors, abnormal expression of lncRNA is caused by gene mutation at brittle gene sites during gene transfer activation (21). More recent studies have identified that SNHG6 promoted growth of nasopharyngeal tumors through targeted regulation of lactotransferrin, and affected glioma cell aging through regulation of cytoplasmic polyadenylation element binding protein 1 gene expression (7,22). These data suggest that lncRNA SnHG6 is closely associated with the occurrence of tumor development.

The downregulation of lncRNA SNHG6 has been associated with a variety of tumors: The expression of lncRNA SNHG6 in breast cancer cells was revealed to inhibit the growth of bone metastasis and lung metastasis in situ tumors, and the survival rate of patients with low expression of lncRNA SNHG6 was significantly decreased compared with that of the high expression group (22). Previous studies have also demonstrated that lncRNA SNHG6 was significantly downregulated in gastric cancer tissues compared with normal tissues, and increased lncRNA SNHG6 may significantly induce cell cycle arrest and inhibition in gastric cancer cells, and inhibit the invasion ability of gastric cancer cells and the ability of tumor and metastasis in vivo (9,23). In addition, other studies have also suggested that lncRNA SNHG6 may inhibit the invasion ability of lung cancer cells (9-11). However, the function and potential mechanism of action of lncRNA SNHG6 in colon cancer remains to be elucidated, in particular how SNHG6 affects cell migratory and invasive capabilities. At present, with the development of sequencing technologies and the progression of investigative studies, a number of lncRNAs have been identified and investigated. It has been demonstrated that SNHG6 affected the migration and invasion of cancer cells by altering the expression of...
epithelial mesenchymal transition; this involves the participation of multiple signaling pathways (9). The present study initially explored the association between SNHG6 and PI3K/AKT/mTOR signaling pathways.

A previous study hypothesized that SNHG6 represented an unusual snoRNA gene, and there is a difference in the expression level of SNHG6. Due to the limitations of the experimental conditions achieved, qPCR was used to detect the expression level of SNHG6. The present study demonstrated that lncRNA SNHG6 inhibited cell proliferation and migration of colorectal cancer cells, and they provide novel insights into the carcinogenesis of colorectal cancer. To characterize the association between ETS1 and SNHG6 in greater detail, correlation between sequences determined by complementarity between sequence bases studies and luciferase tests are required. In addition, it may be useful for the development of a treatment approach for ETS1-activated colorectal cancer. The present study demonstrated that IncRNA SNHG6 inhibited cell proliferation and metastasis by targeting ETS1, and established preliminary associations with the PI3K/AKT/mTOR signaling pathways; future studies investigating the specific mechanisms are required.

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Availability of data and materials

The datasets generated and analyzed during the present study are not publicly available due to further research being performed, but are available from the corresponding author on reasonable request.

Authors’ contributions

SM and ZJ designed the study. XY, and JL performed the experiments. RZ analyzed the data.

Ethics approval and consent to participate

The protocol was approved by the Ethics Committee of Cancer Hospital of China Medical University, and written informed consent was obtained from all study participants.

Patient consent for publication

Written informed consent was obtained from all study participants.

Competing interests

The authors declare that they have no competing interests.

References


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