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LncRNA SNHG3 is responsible for the deterioration of colorectal carcinoma through regulating the miR-370-5p/EZH1 axis

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Abstract. – OBJECTIVE: To illustrate the biological function of long non-coding RNA (lncRNA) SNHG3 in the deterioration of colorectal cancer (CRC) by regulating the miR-370-5p/EZH1 axis.

PATIENTS AND METHODS: SNHG3 levels in fifty pairs of CRC and non-tumor tissues were examined by quantitative real-time polymerase chain reaction (qRT-PCR). Its correlation to tumor staging, lymph node metastasis and prognosis of CRC was analyzed. Cell counting kit-8 (CCK-8) and 5-Ethynyl-2'- deoxyuridine (EdU) assay were conducted to assess the influence of SNHG3 on CRC cell proliferation in vitro. In addition, invasive ability of CRC cells transfected with si-SNHG3 was explored by transwell assay. The binding and regulatory relations in the SNHG3/miR-370-5p/EZH1 axis were ascertained by Dual-Luciferase reporter assay.

RESULTS: SNHG3 was upregulated in CRC tissues and cell lines. Its high level was correlated to advanced tumor staging, positive lymph node metastasis and poor prognosis of CRC. Knockdown of SNHG3 reduced proliferative and invasive rates of SW480 and HT29 cells. The SNHG3/miR-370-5p/EZH1 axis was ascertained. In addition, knockdown of miR-370-5p enhanced proliferative and invasive rates of SW480 and HT29 cells.

CONCLUSIONS: LncRNA SNHG3 induces proliferative and invasive potentials of CRC by regulating the miR-370-5p/EZH1 axis.

Key Words:
CRC, SNHG3, MiR-370-5p, Proliferation, Invasion.

Introduction

Colorectal cancer (CRC) is a frequently diagnosed gastrointestinal malignant tumor[1]. In China, there are about 170,000 new cases each year, and its onset is relatively young, posing a great harm to health[2]. Surgery combined postoperative chemotherapy is the traditional treatment of CRC. The survival of CRC has significantly increased with the development of chemotherapy drugs. The 5-year survival of *in situ* CRC is up to 90%. However, the 5-year survival of metastatic CRC is lower than 50%. Distant metastasis and cancer recurrence are still the main reasons for CRC death[3,4]. A comprehensive management of CRC relies on in-depth understanding of its pathogenesis and molecular mechanism.

Long non-coding RNAs (lncRNAs) cannot encode proteins; they are able to regulate gene expressions at epigenetic, transcriptional or post-transcriptional level[5,6]. Abnormally expressed lncRNAs cause pathological process, especially cancer development[7,8]. A growing number of cancer-associated lncRNAs have been discovered by lncRNA microarray analysis and RNA sequencing[9]. Some lncRNAs display a similar function in different types of cancers, while some have dual-functions[10]. It is reported that lncRNA PVT-1 is upregulated in CRC tissues, which is positively correlated to vascular invasion and lymph node metastasis[11]. LncRNA MALAT1 drives proliferative and metastatic capacities of CRC cells[12].

SNHG (small nucleolar RNA host gene) is a functional lncRNA that mediates the development of malignant tumors[13,14]. SNHG3, also known as U17HG, is located on chr1:28,505,943-28,510,892. It is previously reported that SNHG3

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is of significance in the development of liver cancer[15,16]. SNHG3 has a close relation to lipid metabolism of cells. It is able to mediate metabolic activities of liver cancer cells by inducing the transcription factor SREBP-lc[17]. Our study showed that SNHG3 was upregulated in CRC tissues. The biological function of SNHG3 and the molecular mechanism in CRC were mainly explored.

Patients and Methods

Patients and Samples

A total of 50 pairs of CRC and non-tumor tissues were collected from diagnosed patients in Yantai Affiliated Hospital of Binzhou Medical University from October 2018 to March 219. Samples were pathologically confirmed and stored in liquid nitrogen for use. Tumor staging of CRC conformed to UICC guidelines. This study was approved by the research Ethics Committee of Yantai Affiliated Hospital of Binzhou Medical University and complied with the Helsinki Declaration. Informed consent was obtained from each patient.

Cell Culture

CRC cell lines (SW480, SW620, HCT8, HT29) and human colonic epithelial cell line (NCM460) were provided by American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) at 37°C, 5% CO_2.

Cell Transfection

Transfection plasmids were synthesized by Ribobio (Guangzhou, China). Cells were seeded in a 6-well plate and cultured to 80% density. After 48-h transfection using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), cells were collected for verifying transfection efficacy and functional experiments.

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

Tissue or cell samples were processed by TRIzol (Invitrogen, Carlsbad, CA, USA) for isolating RNAs. After purification, qualified RNAs were reversely transcribed to complementary deoxyribose nucleic acids (cDNAs) and subjected to qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). Relative levels of PCR products were calculated by $2^{-\Delta\Delta Ct}$ and normalized to that of glyceraldheyde 3-phosphate dehydrogenase (GAPDH). Primer sequences were listed as follows. SNHG3 (F: 5'-TACTGGCTGCGCACTTCG-3', R: 5'-TACCCTGCACAAACCCGAAA-3'); miR-370-5p (F: 5'-GAGGGTTAATGCTAATCGTGATAGG-3', R: 5'-GCACAGAATCAACACGACTCACTAT-3'); EZH1 (F: 5'-CTCTACCTCCACCATGCCAAGT-3', R: 5'-GCTGCGCTGATAGACATCCA-3'); U6 (F: 5'-CTCGCTTCGGCAGCACA-3', R: 5'-AACGCTTCACGAATTTGCGT-3'); GAPDH (F: 5'-GGAATCCACTGGCGTCTTCA-3', R: 5'-GGTTCACGCCCATCACAAAC-3').

Cell Counting Kit-8 (CCK-8) Assay

Cells were seeded in a 96-well plate with 2×10^3 cells suspended in 200 μL of medium per well. They were induced with 10 μL of CCK-8 solution (Dojindo, Kumamoto, Japan) per well at the indicated time points. After cell culture in the dark for 2 h, absorbance at 450 nm was detected for plotting cell viability curves.

Transwell Assay

1×10^4 cells suspended in serum-free medium were seeded in a transwell chamber. On the bottom of the chamber, 200 mg/mL Matrigel diluted in serum-free medium at a ratio of 1:6 was pre-coated and dried overnight. Medium containing 10% FBS was added to the bottom as an inducer. After 24-h cell culture, invasive cells to the bottom were fixed, and dyed in 0.2% crystal violet. They were captured in 5 random fields per well for cell counting.

5-Ethynyl-2'-deoxyuridine (EdU) Assay

Cells were seeded in a 12-well plate (5×10^4 cells / well), labeled by 50 μM EdU (RiboBio, Guangzhou, China) for 2 h, and dyed using Ado-Lo and 4',6-diamidino-2-phenylindole (DAPI) in the dark. EdU-labeled cells were captured for calculating EdU-positive rate.

Dual-Luciferase Reporter Assay

Luciferase vectors were constructed based on the predicted binding site in miR-370-5p 3'UTR. They were co-transfected into cells with miR-370-5p mimics or NC for 48 h. Cells were induced with 35 μL of luciferase substrate per well for 10 min. Luciferase activity (Promega, Madison, WI, USA) was measured for three times and the average value was calculated.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism (Version X; La Jolla, CA, USA) were used for statistical analyses and figure editing, respectively. Differences between groups were compared by the *t*-test. Kaplan-Meier method and log-rank test were introduced for survival analysis. Correlation between expression levels of two genes in osteosarcoma tissues was assessed by Pearson correlation test. $p<0.05$ was considered as statistically significant.

Results

Clinical significance of SNHG3 in CRC

QRT-PCR data showed that SNHG3 was upregulated in CRC tissues than normal ones (Figure 1A). Analysis on pathological data of recruited CRC patients uncovered that SNHG3 level was higher in T3-T4 CRC patients in comparison to T1-T2 patients (Figure 1B). CRC patients with the absence of lymph node metastasis expressed a lower level of SNHG3 (Figure 1C). Moreover, Kaplan-Meier curves showed a poor overall survival in CRC patients expressing high level of SNHG3 (Figure 1D). SNHG3 was upregulated in CRC cell lines as well (Figure 1E).

Knockdown of SNHG3 Reduced Proliferative and Invasive Rates of CRC Cells

SNHG3 knockdown model was established in SW480 and HT29 cells by transfection of si-SNHG3 (Figure 2A). Cell viability was detected to be declined by knockdown of SNHG3 in SW480 and HT29 cells (Figure 2B, 2C). As expected, EdU-positive rate decreased in CRC cells transfected with si-SNHG3 (Figure 2D, 2E). Transwell assay showed that knockdown of SNHG3 in SW480 and HT29 cells reduced invasive cell number (Figure 2F, 2G).

SNHG3 Could Bind to MiR-370-5p and Downregulated its Level

Based on the predicted binding site within miR-370-5p, wild-type and mutant-type SNHG3 vectors were constructed for Dual-Luciferase reporter assay. Overexpression of miR-370-5p decreased Luciferase activity in the wild-type vector, rather than the mutant-type one, suggesting that SNHG3 could bind to miR-370-5p (Figure 3A-3C). After verifying the transfection efficacy of miR-370-5p inhibitor, it is found that SNHG3 was upregulated by knockdown of miR-370-5p in CRC cells (Figure 3D, 3E). Similarly, miR-370-5p was upregulated by transfection of si-SNHG3 (Figure 3F). MiR-370-5p was downregulated in CRC tissues, and displayed a negative correlation to that of SNHG3 (Figure 3G, 3H).

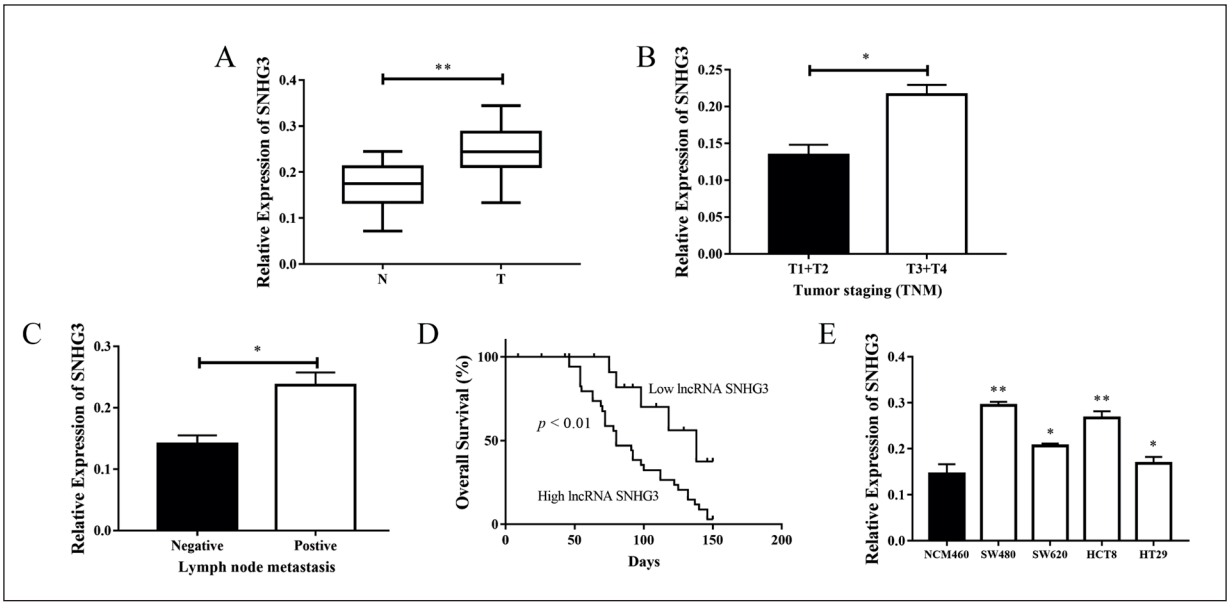

Figure 1. Clinical significance of SNHG3 in CRC. **A,** SNHG3 was upregulated in CRC tissues; **B,** Correlation between SNHG3 and tumor staging of CRC; **C,** Correlation between SNHG3 and lymph node metastasis of CRC; **D,** Correlation between SNHG3 and overall survival of CRC; **E**, SNHG3 was upregulated in CRC cell lines. *$p < 0.05$, **$p < 0.01$.

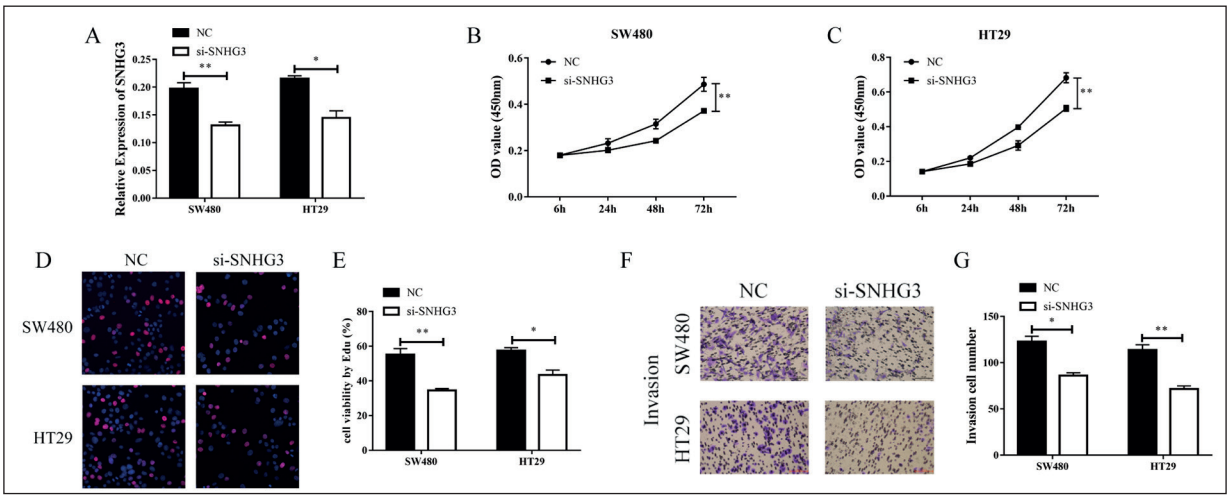

Figure 2. Knockdown of SNHG3 reduced proliferative and invasive rates of CRC cells. **A,** Transfection of si-SNHG3 significantly downregulated SNHG3 in SW480 and HT29 cells; **B, C,** Transfection of si-SNHG3 significantly decreased viability in SW480 and HT29 cells; **D, E,** Transfection of si-SNHG3 significantly decreased EdU-positive rate in SW480 and HT29 cells; (magnification: 200×) **F, G,** Transfection of si-SNHG3 significantly decreased invasive rate in SW480 and HT29 cells. (magnification: 200×); *$p < 0.05$, **$p < 0.01$.

Knockdown of MiR-370-5p Enhanced Proliferative and Invasive Rates of CRC Cells

We next explored the role of miR-370-5p in mediating CRC development. CCK-8 and EdU assay demonstrated that knockdown of miR-370-5p enhanced proliferative rate of SW480 and HT29 cells (Figure 4A-4D). Invasive rate was elevated by transfection of miR-370-5p inhibitor in CRC cells (Figure 4E, 4F).

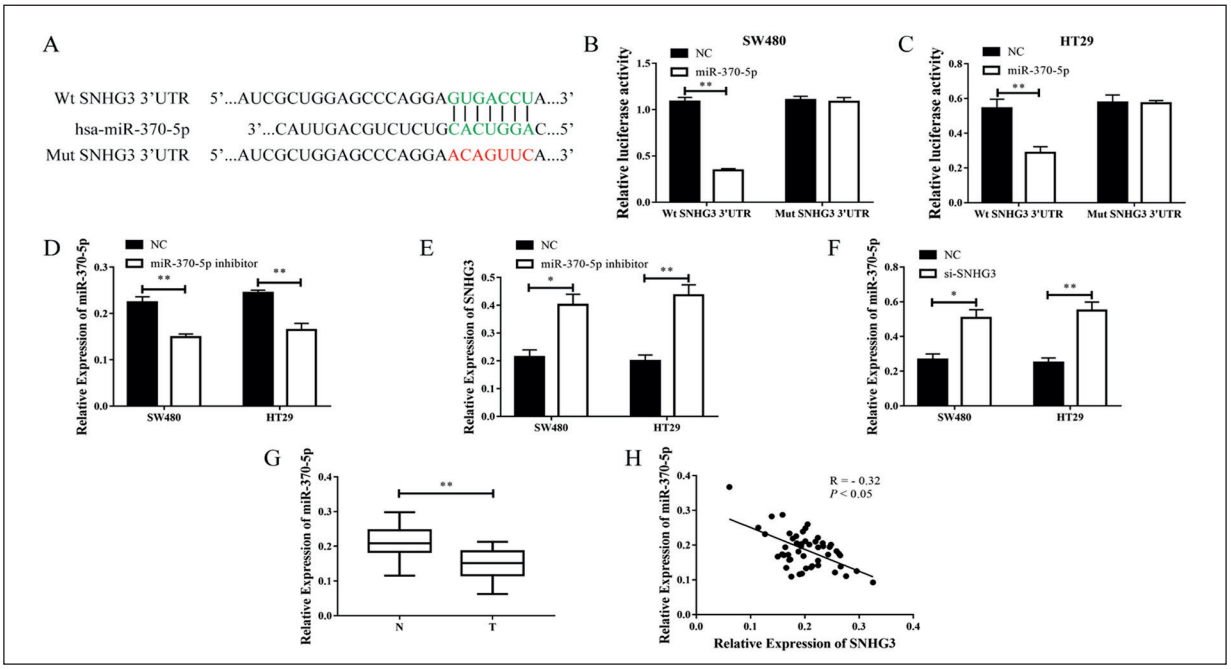

Figure 3. SNHG3 could bind to miR-370-5p and downregulated its level. **A,** Binding sites in SNHG3 and miR-370-5p; **B, C,** SNHG3 directly bound to miR-370-5p in SW480 and HT29 cells; **D,** Transfection of miR-370-5p inhibitor significantly downregulated miR-370-5p in SW480 and HT29 cells; **E,** Transfection of miR-370-5p inhibitor significantly upregulated SNHG3 in SW480 and HT29 cells; **F,** Transfection of si-SNHG3 significantly upregulated miR-370-5p in SW480 and HT29 cells; **G,** MiR-370-5p was downregulated in CRC tissues; **H,** MiR-370-5p was negatively correlated to SNHG3 in CRC tissues. *$p < 0.05$, **$p < 0.01$.

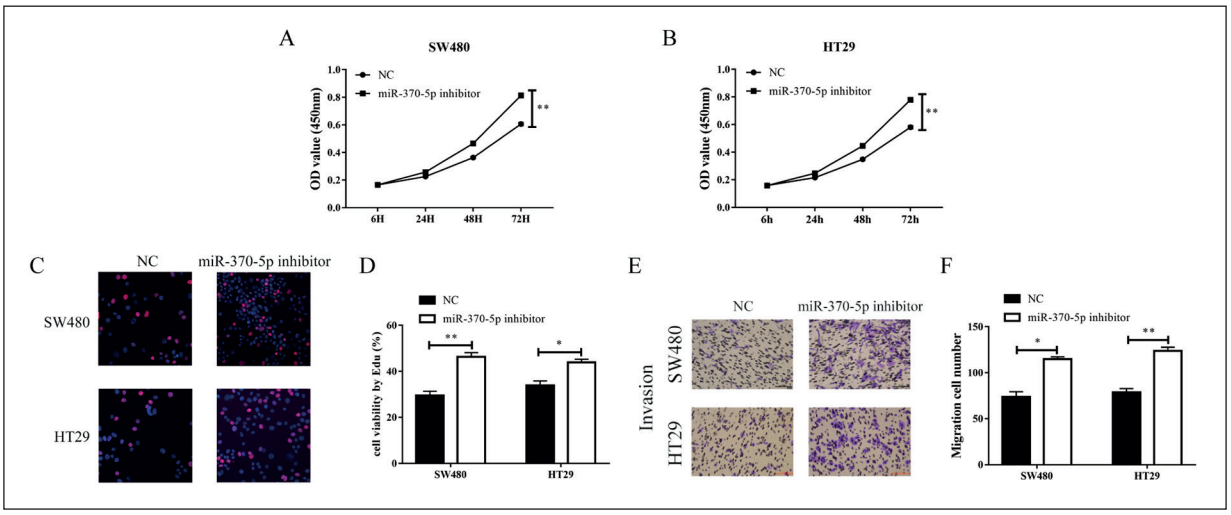

Figure 4. Knockdown of miR-370-5p enhanced proliferative and invasive rates of CRC cells. **A, B,** Transfection of miR-370-5p inhibitor significantly increased viability in SW480 and HT29 cells; **C, D,** Transfection of miR-370-5p inhibitor significantly increased EdU-positive rate in SW480 and HT29 cells; (magnification: 200×) **E, F,** Transfection of miR-370-5p inhibitor significantly increased invasive rate in SW480 and HT29 cells. (magnification: 200×); *$p < 0.05$, **$p < 0.01$.

EZH1 was the Downstream Target of MiR-370-5p

In the same way, EZH1 was identified to be the downstream target of miR-370-5p (Figure 5A-5C). In CRC cells, EZH1 was upregulated by knockdown of miR-370-5p, but downregulated by knockdown of SNHG3 (Figure 5D, 5E).

Discussion

The incidence and mortality of CRC show significant increases in recent years[18,19]. Most of death cases of CRC are attributed to distant metastases and cancer recurrences[20]. Currently, the etiology of CRC remains unclear[21]. Clarifying the

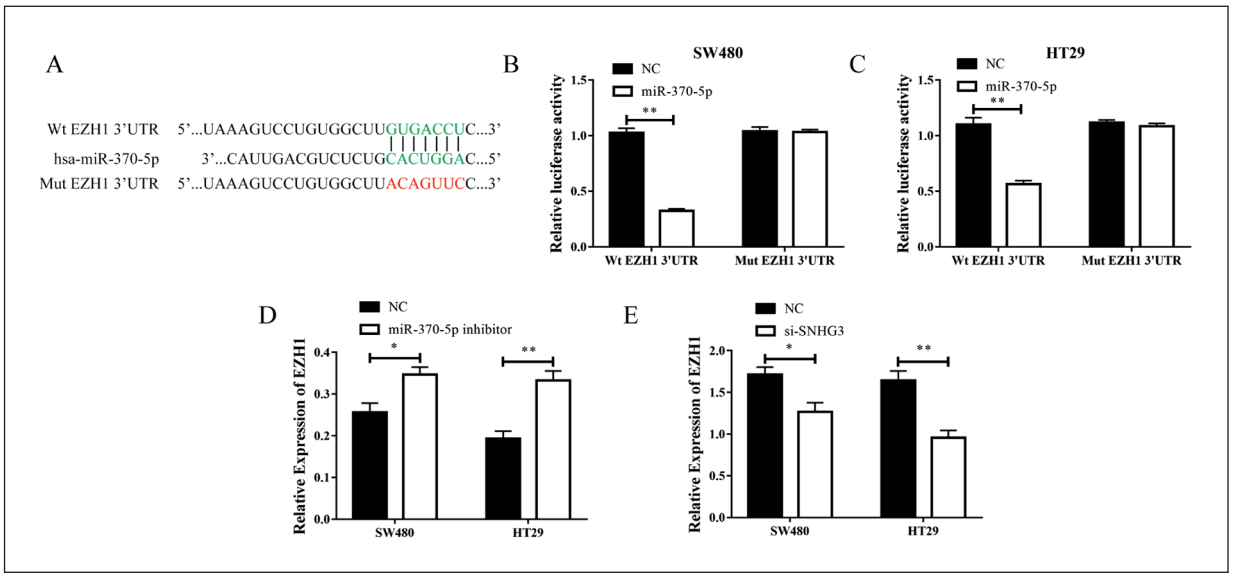

Figure 5. EZH1 was the downstream target of miR-370-5p. **A,** Binding sites in EZH1 and miR-370-5p; **B, C,** SNHG3 directly bound to miR-370-5p in SW480 and HT29 cells; **D,** Transfection of miR-370-5p inhibitor significantly upregulated EZH1 in SW480 and HT29 cells; **E,** Transfection of si-SNGH3 significantly downregulated EZH1 in SW480 and HT29 cells. *$p < 0.05$, **$p < 0.01$.

etiology and pathogenesis of CRC and seeking for anti-cancer targets are of significance to prevention and treatment of CRC.

In our research, we first found that SNHG3 was upregulated in CRC tissues. By analyzing pathological data of recruited CRC patients, SNHG3 level was correlated to advanced tumor staging, positive lymph node metastasis and poor prognosis. In addition, SNHG3 was also highly expressed in CRC cell lines. Knockdown of SNHG3 reduced proliferative and invasive rates of SW480 and HT29 cells. In the previous studies, SNHG3 was confirmed to promote disease progression in diverse cancers by acting as a ceRNA. SNHG3 accelerated the proliferation and invasion of non-small cell lung cancer by downregulating miR-340-5p[22]. In addition, SNHG3 promotes the growth of ovarian cancer cells by targeting miR-339-5p/TRPC3 Axis[23]. In CRC, SNHG3 could promote the growth and metastasis by regulating miR-539/RUNX2 axis[24]. In addition, SNHG3 was also found to promote development of CRC by function as a ceRNA to binding miR-182-5p to release c-Myc[25].

The previous researches indicated that SNHG3 may act as a ceRNA to regulate the target genes. In our research, we found that miR-370-5p was a target binding miRNA of SNHG3 through Dual-Luciferase assay. Through literature review, miR-370-5p was reported to inhibit the progression of breast cancer progression by targeting LUC7L3[26]. In our research, miR-370-5p was remarkably downregulated in CRC tissues, and it was negatively correlated to SNHG3 level. Knockdown of miR-370-5p was able to enhance proliferative and invasive abilities of CRC cells.

At last, through Dual-Luciferase assay, EZH was found to be the target gene of miR-375-5p. Enhancer of zeste homolog 1 (EZH1) belongs to PcG family, which is a human homology of zeste gene enhancer in Drosophila melanogaster. EZH1 is responsible for the development of fetal hematopoietic system, central and peripheral nervous system by forming PcG protein complexes[27]. It also participates in histone methyl transfer process, maintenance of pluripotency of stem cells, cancer development, etc.[28,29]. In colon cancer patients, EZH1 level is correlated to the incidence of lung metastases[30].

In our research, it is concluded that SNHG3 was responsible for the deterioration of CRC by regulating the miR-370-5p/EZH1 axis, proving a new therapy for CRC progression.

Conclusions

LncRNA SNHG3 induces proliferative and invasive potentials of CRC by regulating the miR-370-5p/EZH1 axis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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