LncRNA SNHG22 Sponges miR-128-3p to Promote Progression of Colorectal Cancer through Upregulating E2F3

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Abstract

Background: Long non-coding RNA (lncRNA) termed small nucleolar RNA host gene 22 (SNHG22) has been reported as a crucial regulator in several types of human cancers. In this study, we aimed to evaluate the function and mechanism of SNHG22 in colorectal cancer (CRC) progression.

Methods: Quantitative RT-PCR (qRT-PCR) was used to detect the expression of SNHG22 in adenoma, tumor tissues (TTs), and adjacent nontumorous tissues (ANTs). The biological behaviors of SNHG22 in CRC cell lines were explored both in vitro (CCK-8 assay, flow cytometry, wound scratch, and transwell assays) and in vivo (nude mouse xenograft model). The interaction between SNHG22 and miR-128-3p, and the target genes of miR-128-3p were explored by online tools, qRT-PCR, western blot, and dual-luciferase reporter assay.

Results: SNHG22 expression was gradually upregulated in ANTs, adenoma, and TTs. High expression levels of SNHG22 were significantly related to advanced clinicopathological factors and worse survival in patients with CRC. SNHG22 knockdown markedly prohibited CRC cell proliferation, migration, and invasion; and drove cell apoptosis in vitro; and hindered tumor growth in vivo. Mechanistic investigation showed that SNHG22 could bind to microRNA-128-3p (miR-128-3p) and attenuate its inhibitory effects on the expression levels and activity of E2F3. Rescue experiments exhibited that miR-128-3p inhibition or E2F3 upregulation can offset the functions of SNHG22 knockdown in CRC cells.

Conclusion: Our findings support the existence of an interactive regulatory network of SNHG22, miR-128-3p, and E2F3 in CRC cell lines, indicating that the SNHG22/miR-128-3p/E2F3 axis is a novel diagnostic and therapeutic target in CRC.

Introduction

Colorectal cancer (CRC) represents one of the most common malignancies worldly. Every year, over 1.3 million people are diagnosed with CRC in the world, of which nearly 0.7 million succumb to this disease.¹ Only about fifty percent of patients with CRC will be alive at five years after diagnosis,² although some improvements in early diagnosis and systemic therapies have been made. The pathogenesis and progression of CRC is a complex process, and the potential mechanism remains unclear yet.

Long non-coding RNAs (lncRNAs) are a class of non-encoding RNA longer than 200 nucleotides. Recent researches have indicated that many lncRNAs exert an important role in almost all physiological and pathological processes, including embryonic stem cell, carcinogenesis, and cancer metastasis.^{3,4} The lncRNAs have been reported to function as oncogenes or tumor suppressor gene, participating in CRC tumorigenesis and progression. For example, a report from Xu et al. showed that lncRNA MIR17HG drove tumor development and metastasis in colorectal cancer cells via enhancing the expression of NF- κ B/RELA.⁵ HOTAIR enhanced CRC cells migration and drug resistance via miR-203a-3p dependent Wnt/

β -Catenin signaling pathway. As an oncogene, LncRNA H19 accelerated cell proliferation and metastasis in CRC by acting on Wnt signaling.⁶

Small nucleolar RNAs (snoRNA), a subgroup of ncRNAs with 60–300 nucleotides in length, contributes to the tumorigenesis and metastasis in diverse human cancers. Most snoRNAs are encoded in the introns of snoRNA host genes (SNHG).⁷ Specifically, many SNHGs are shown in the carcinogenesis and progression in CRC. For example, overexpressed SNHG7 is shown tumor tissues (TTs) as compared with adjacent noncancerous tissues (ANTs) in CRC, and this high expression is related to aggressive pathological characteristics, such as tumor size, TNM stage, and distant metastasis, as well as dismal survival.^{8,9} SNHG22, located on chromosome 8q21.1, has 2157 nucleotides in length.¹⁰ Several reports have found that SNHG22 contributed to cell growth, migration, invasion, and chemotherapy resistance in epithelial ovarian carcinoma (EOC), papillary thyroid cancer (PTC) and clear cell renal cell carcinoma (ccRCC).^{11–13} Besides, upregulated SNHG22 was shown in TTs as compared with that in the ANTs, and whose high expression indicated poor prognosis in the three types of human malignancies.^{11–13} To our knowledge, the biological role and expression patterns of SNHG22 have not been examined in human CRC.

In the current study, we investigated SNHG22 expression in adenoma and CRC tissues and evaluated its clinical significance. Moreover, the biological behaviors of SNHG22 in the migratory and invasive phenotype of CRC cell lines were explored in vitro as well as in vivo. We also investigated the underline mechanisms involved in the pro-oncogenic effects of SNHG22 on CRC in detail.

Material And Methods

2.1 Tissue samples and Cell

We obtained 93 paired CRC tissues and matched ANTs from patients who underwent surgery at the First Affiliated Hospital, Zhengzhou University. Additional fresh specimens were collected from colorectal adenoma (CRA) patients (n = 33) who underwent colonoscopy. The diagnosed with CRA or CRC according to histopathologic evaluation. All specimens were snap-frozen in liquid nitrogen quickly and stored at – 80 °C until required. The study was approved by the Ethics Committee of Zhengzhou University. Patients with any history of other cancers, receiving preoperative radiotherapy or chemotherapy, were excluded. The clinical characteristics of all patients are listed in **Supplementary Table S1**.

Human CRC cell lines, Caco2, LS174T, LoVo, SW480, HT-29, and SW620 were available from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). These cell lines were maintained in RPMI-1640 medium (Gibco) containing 10% fetal bovine serum (FBS; Gibco) in a humidified incubator (37°C, 5% CO₂). Human normal colon epithelial cell line FHC were purchased from Mingzhou Company (MZ-0713, Zhejiang, China), which was cultured in 90% DMEM (Gibco) supplemented with 20% FBS (Gibco).

2.2 Cell transfection

Small interfering RNAs (siRNAs) against SNHG22 (sh#1, sh#2) or E2F3 (siE2F3), as well as overexpression vectors, pcDNA3.1/Control (Vector), pcDNA3.1/SNHG22 (SNHG22), and pcDNA3.1/E2F3 (E2F3), were purchased from Gene Pharma (Shanghai, China). Plasmid with a non-targeting sequence was used as a negative control (siNC). The microRNA mimics, inhibitor, and negative control (NC mimic and NC inhibitor) were purchased from Ribio (Guangzhou, China). In vivo experiments, vectors containing short hairpin RNAs (shRNA) targeting SNHG22 (shSNHG22), sh#1, and sh#2 were subcloned into Lv5 lentiviruses (GenePharma, Shanghai, China) and infection into LoVo cells to generate Lv-sh#1 and Lv-sh#2. Before transfection, the cells were cultured for 24 h. Then, the cells were transiently transfected with the corresponding vector using Lipofectamine 3000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.3 Cell proliferation

Cell proliferation ability was examined using a cell counting kit-8 (CCK-8, Beyotime Institute of Biotechnology, Shanghai, China). The absorbance in each well was measured at 0, 1, 2, and 3 d after transfection by a microplate reader (Bio-Rad, Hercules, CA, USA).

2.4 Flow cytometry

Cell cycle distribution was detected using flow cytometry. After CRC cells were harvested, washed, and resuspended in DNA staining solution, we analyzed the fractions in the G0/G1, S, and G2/M phases. Cell apoptosis was detected using an Annexin VFITC/ propidium iodide (PI) Apoptosis Detection kit (MultiSciences Biotech Co., Ltd., Hangzhou, China). After CRC cells (5×10^4 /well) were seeded and resuspended in 12well plates, Annexin VFITC (5 μ l) and PI (100 μ l) were added to each reaction system for 6 minutes. Immediately, flow cytometric assays were conducted on a flow cytometry (EPICS, Beckman, CA, USA).

2.5 Wound-scratch

Cell migration ability was examined using a wound-scratch assay. Briefly, CRC cells were seeded into a 96-well plate and grown to confluence. To produce a clear line in the wells, we utilized a sterile 20 μ L pipette tip. Cells were photographed to record the wound width (0 h). After 24 h, pictures were retaken. Migration distance was counted at the time of 0 h and 24 h.

2.6 Cell invasion

Transwell inserts used for invasion assay were pre-coated with Matrigel (BD Falcon, San Jose, CA). Briefly, 2×10^5 cells in 200 μ L serum-free medium were suspended into the upper chamber, and 700 μ L medium with 15% FBS was added into the lower chamber. Following 24 h incubation, the chamber membranes were fixed in 4% paraformaldehyde and stained with crystal violet for 18 minutes. The cells onto the membrane were counted in five random fields.

2.7 Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from frozen tissues and exponentially growing cells using TRIzol (Invitrogen, Carlsbad, CA, USA). Nuclear and cytoplasmic RNA was separated using the PARIS Kit (Thermo Fisher Scientific, Waltham, Mass., USA). The cDNA was synthesized from total RNA (1000 ng) using a reverse transcriptase cDNA synthesis kit (Toyobo). The expression levels were measured using SYBR Premix Ex Taq II (Takara) on the CFX96 sequence detection system (Bio-Rad). The primer sequences were shown in **Supplementary Table S2**. qRT-PCR data were calculated by the $2^{-\Delta\Delta CT}$ methods using actin and U6 as the internal control.¹⁴

2.8 Western blot

Total proteins were extracted using RIPA buffer (Thermo Fisher Scientific). Proteins (20 µg) were separated with 10% SDS-PAGE gels and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, USA). After blocking, the membranes were blotted with the primary antibody against E2F3 (ab50917, Abcam, England) for 16 h and then incubated with secondary antibodies for 2 h. Immunoreactive bands were detected using chemiluminescence (Pierce Chemical Co., Rockford, IL). β-actin (ab8227, Abcam, England) acted as a loading control.

2.9 Luciferase reporter

The predicted binding sites of miR-128-3p with SNHG22-3' untranslated region (UTR) or E2F7-3'UTR were obtained from Starbase 3.0. The mutant type (mut) SNHG22 and E2F7 3' UTR luciferase reporter vectors were available by using a Mutagenesis Kit (QIAGEN, California, USA). The binding and mutant sequences were respectively cloned into the psiCHECK2 vector (Promega, Madison, WI, USA). CRC cells and 293 T cells in 96-well plates were co-transfected with the wt/mut plasmid and miR-128-3p or NC using Lipofectamine RNAi MAX (Invitrogen, USA). After transfection for 36 h, cells were collected and tested using a Dual-Luciferase Assay System (Promega, Madison, WI) following the manufacturer's instructions. PRL-TK was used as internal control.

2.10 RNA immunoprecipitation (RIP)

RIP was carried out using the EZ-Magna RIP Kit (Millipore) and an Ago2 antibody (Abcam). CRC cell lines (2×10^7) were lysed in RIP buffer and centrifuged at 10 000 *g* for five min. Cell lysis was incubated with magnetic beads conjugated with anti- Argonaute (Ago2; ab32381, Abcam) or IgG for 3 hours. After extensive washing using an elution buffer at 65 °C for 10 minutes, the purified RNA was analyzed by qRT-PCR.

2.11 Animal experiments

Animal experimental procedures were approved by the Animal Ethics Committee of Zhengzhou University (He' nan, China). A total of 15 male immune-deficient BALB/c nude mice (Four-week-old; Shanghai Experiment Animal Centre; Shanghai, China) were maintained under specific pathogen-free conditions and randomly subdivided into three subgroups. The Lv-shNC, Lv-sh#1, and Lv-sh#2 vectors stably

transfected LoVo cells were harvested. Cells (6×10^6) were injected subcutaneously into the dorsal flank regions of each mouse. Tumor growth was measured every 5 days and was calculated according to the formula $\text{volume} = (\text{width}^2 \times \text{length})/2$. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the NIH (Bethesda, MD).

2.12 Immunohistochemistry (IHC)

After sections were blocked in 3% hydrogen peroxide, they were ready for IHC. The slides were incubated with polyclonal antibodies to Ki-67 (1: 800, Abcam) using the DAKO Envision system (DAKO, Carpinteria, California) as described previously.¹⁵

2.13 Bioinformatics and statistical analysis

Data mining analyses were performed based on the databases, including Lnclocator,¹⁶ CPAT (<http://lilab.research.bcm.edu/cpat/index.php>), LNCipedia (<https://lncipedia.org>), miRcode (www.mircode.org), miranda (www.microrna.org), StarBase 3.0 (<http://starbase.sysu.edu.cn/>), and UALCAN (<http://ualcan.path.uab.edu/index.html>). All data were presented as mean $\pm$ standard deviation (SD) from triplicate experiments. The survival curves are conducted using the Kaplan-Meier method and analyzed using the log-rank test. Crude and adjusted hazard ratios (HR) and 95% confidence intervals (CI) were calculated using Cox proportional hazards modeling. All statistical analyses were conducted by using the PASW Statistics 19.0 software program (SPSS, Chicago, IL, USA) and GraphPad Prism version 8.0 (GraphPad Inc., La Jolla, CA, USA). A P -value < 0.05 was considered statistically significant.

Results

3.1 SNHG22 is upregulated in CRC tissues and cell lines and correlates with poor prognosis in CRC patients

To investigate SNHG22 expression levels in CRC, we conducted the qRT-PCR analysis to examine the SNHG22 levels in TT and ANT colon samples (93 pairs) and human CRA samples ($n = 32$). Results showed that the expression of SNHG22 in TTs colon tissues was significantly higher than those in adenoma tissues and ANTs (Fig. 1A). Additionally, correlation analysis suggested that high SNHG22 expression was significantly related to advanced T stage ($P < 0.001$; Fig. 1B), lymph node metastasis ($P = 0.0042$; Fig. 1C), distant metastasis ($P < 0.001$; Fig. 1D), advanced clinical stage ($P = 0.042$; Fig. 1E), and poor disease-free survival (DFS) and overall survival (OS; $P = 0.006$ and 0.008 , respectively; Fig. 1F-G). Interestingly, data from TCGA also confirmed our results (Fig. 1H-I). Multivariate cox regression analysis validated SNHG22 expression levels as an predictor for DFS (hazard ratio [HR] = 1.68, 95% confidence interval [CI]: 1.04–2.71) and OS (HR = 1.45, 95%CI: 1.02–2.06; Table 1). High SNHG22 expression was also confirmed in CRC cell lines (LS174T, LoVo, Caco2, SW480, HT-29, and SW620) as compared with FHC cell lines (Fig. 1J). These results exhibited that SNHG22 was indeed highly expressed in CRC tissues and cell lines, and was associated with poor survival of CRC cases. Also, the coding potential of SNHG22

was calculated using the online assessment tools (CPAT and LNCipedia). The results showed that the coding probability of SNHG22 is very low (**Supplementary Fig. S1**).

Table 1
Cox's proportional hazards regression models for overall and recurrence-free survival among CRC Patients

Parameters	Univariate analysis		Multivariate analysis	
	HR (95%CI)	P	HR (95%CI)	P
Disease-free survival				
Location, rectum vs. colon	1.98(0.85–4.60)	0.112	1.67(0.71–3.90)	0.237
T stage, T3-4 vs. T1-2	2.13(1.05–4.30)	0.035	1.66(1.02–2.70)	0.041
Node involvement, yes vs. no	2.35(1.07–5.13)	0.033	1.27(1.00-1.61)	0.048
M stage, M1 vs. M0	2.56(1.23–5.33)	0.012	1.51(1.04–2.20)	0.031
Tumor differentiation, poor vs. well + moderate	1.49(0.95–2.34)	0.084	1.14(0.88–1.48)	0.321
CEA, > 5 vs. <5 ng/ml	1.40(0.68–2.86)	0.362		
Margin, positive vs. negative	1.32(0.61–2.84)	0.483		
Chemotherapy yes vs. no	1.34(0.57–3.15)	0.501		
SNHG22, high vs. low	2.47(1.13–5.41)	0.023	1.68(1.04–2.71)	0.034
Overall survival				
Location, rectum vs. colon	2.19(1.01–4.75)	0.047	1.81(0.83–3.96)	0.138
T stage, T3-4 vs. T1-2	1.59(1.00-2.53)	0.050	1.33(0.97–1.82)	0.077
Node involvement, yes vs. no	1.91(1.12–3.26)	0.018	1.57(1.06–2.33)	0.025
M stage, M1 vs. M0	1.98(1.24–3.15)	0.004	1.49(1.08–2.05)	0.014
Tumor differentiation, poor vs. well + moderate	1.39(0.66–2.93)	0.395		
CEA, > 5 vs. <5 ng/ml	1.06(0.54–2.09)	0.868		

Parameters	Univariate analysis		Multivariate analysis	
	HR (95%CI)	P	HR (95%CI)	P
Margin, positive vs. negative	0.97(0.45–2.08)	0.930		
SNHG22, high vs. low	2.21(1.08–4.55)	0.031	1.45(1.02–2.06)	0.039

NOTE: Tumor classification and stage were referred to the 7th edition of UICC (2009) on cancer staging system.

3.2 Upregulation of SNHG22 stimulates proliferation, migration, invasion and inhibits apoptosis of CRC cells in vitro

To further investigate the role of SNHG22 in the progression of CRC cell, we used Caco2 and LS147T cell lines with low SNHG22 expression levels in the following gain-of-function experiments. SNHG22 expression was significantly enhanced after transfecting with pcDNA/SNHG22 in Caco2 and LS147T cells (**Supplementary Fig.s2**). Functionally, the upregulation of SNHG22 promoted the proliferation, G2/M phase arrest, but decreased the rate of early apoptotic cells in both cell lines (Fig. 2A-F). Furthermore, wound-healing and transwell assays demonstrated a marked enhancement in the proliferation and invasive ability of Caco2 and LS147T cells with SNHG22 overexpression (Fig. 2G-J). These data implicated that overexpression of SNHG22 promoted CRC cell proliferation, migration, invasion, but inhibited apoptosis.

3.3 Knockdown of SNHG22 inhibits cell proliferation, migration, invasion, but stimulates apoptosis of CRC cell lines in vitro

To determine whether SNHG22 silencing could affect the biologic function of CRC cells, we used two siRNAs targeting the SNHG22 (si#1 and si#2) for the knockdown experiments. QRT-PCR assay confirmed the knockdown efficiency of both siRNAs in CRC cell LoVo (**Supplementary Fig.s3**). The CCK-8 and flow cytometry assay demonstrated a marked decrease in the proliferation, G2/M phase arrest, but an increase in the percentage of early apoptosis in LoVo cells (Fig. 3A-C). Further wound-healing and transwell assays showed that silencing SNHG22 attenuated the migratory and invasive ability of LoVo cells (Fig. 3D-E). These data implicated that knockdown of SNHG22 inhibited growth, migration, invasion of CRC cell lines.

3.4 Knockdown of SNHG22 inhibits tumor xenograft growth of CRC *in vivo*

We then conducted a tumor xenograft assay to confirm the roles of SNHG22 in the growth of CRC *in vivo*. As exhibited in Fig. 4A-C, compared with the Lv-siNC group, the size, volumes, and weight of tumors in mice of the Lv-si#1 and Lv-si#2 group were remarkably reduced ($P < 0.01$). Furthermore, qRT-PCR assay verified the decreased levels of SNHG22 expression in tumors from the Lv-si#1 and Lv-si#2 group, respectively, compared with control tumors (Fig. 4D). The expression levels of Ki-67 staining by IHC were decreased in tumors from the Lv-si#1 and Lv-si#2 groups compared to that in tumors from the control group (Fig. 4E-F).

3.5 SNHG22 functions as a ceRNA to regulate miR-128-3p expression in CRC

Because subcellular localization of lncRNA could indicate its function, results from the IncLocator¹⁶ indicated that SNHG22 was preferentially localized in the cytoplasm (**Supplementary Fig. s4**). Further cellular fractionation experiments also confirmed this in CRC cells (Caco2, LS147T, and LoVo; Fig. 5A). Based on the online softwares, such as Targetscan7.1, StarBase v3.0, miRcode, and miRanda, we found that SNHG22 contained complementary binding sites to miR-128-3p seed regions (Fig. 5B). To test this hypothesis, we first performed RIP experiments with anti-Ago2 in Caco2, LS147T cells, and observed enrichment of SNHG22 with the Ago2 antibody (Fig. 5C). To further explore whether SNHG22 was regulated by miR-128-3p, we constructed dual-luciferase reporters containing wt or mut putative binding sites of SNHG22 transcripts, respectively. Markedly lower luciferase activity was discovered when SNHG22 wt and miR-128-3p mimic were co-transfected into 293T cells. However, no statistical changes in the luciferase activity were observed in the reporter with the mutated binding sites. These results were also confirmed in Caco2, LS147T cells (Fig. 5D). Furthermore, qRT-PCR assay showed that the up-regulation of SNHG22 dramatically decreased, while silencing SNHG22 boosted, the expression levels of miR-128-3p in CRC cells ($P < 0.01$, Fig. 5E-5F). Expression of SNHG22 had a significant inverse relationship with miR-128-3p expression in human CRC tissues from the Zhengzhou cohort (Spearman's $R = -0.3857$, $P < 0.001$; Fig. 5G). The findings demonstrated that SNHG22 acted as a sponge of miR-128-3p in CRC.

3.6 MiR-128-3p targets and regulates E2F3

We then performed an analysis of 5 independent algorithmic programs to define putative targets, and 25 common predicted targets were identified (Fig. 6A). Among these, E2F3 was selected because reports have indicated that E2F3 could enhance CRC progression and be associated with tumor status based on TCGA-COAD dataset (Fig. 6B **and supplementary fig. s5A-B**). We showed that the up-regulation of miR-128-3p dramatically decreased, while silencing miR-128-3p enhanced, the expression levels of E2F3 mRNA and protein in Caco2 and LS147T cells (Fig. 6C-F). Moreover, Spearman's correlation analysis found a significant inverse relationship between the expression of E2F3 mRNA and miR-128-3p in human

CRC tissues from the Zhengzhou cohort (Fig. 6G). To determine if E2F3 is an authentic target of miR-128-3p, we generated luciferase reporter gene constructs wherein the 3'-UTR sequences of E2F3 were fused with the renilla luciferase coding sequence (Fig. 6H). We demonstrated that the up-regulation of miR-128-3p significantly decreased, while miR-128-3p silencing increased, the luciferase activity of E2F3 wt 3'-UTR (Fig. 6I). Nevertheless, changing miR-128-3p expression showed no significant effects on mut construct (Fig. 6I). In addition, the expression of E2F3 mRNA had a significant positive relationship with SNHG22 in human CRC tissues from the Zhengzhou cohort (Fig. 6J). Our data suggested that E2F3 could be a direct target of miR-128-3p in CRC cells.

3.7 SNHG22 exerts its function by inhibiting the miR-128-3p/E2F3 axis in CRC cells

We then conducted functional rescue experiments to examine whether miR-128-3p/E2F3 axis mediated the biological roles of SNHG22 in CRC cells. Overexpression of SNHG22 or E2F3 stimulated the proliferative, migratory, and invasive capacity of Caco2 cells, and these influences were reversed by the upregulation of miR-128-3p in caco2 cells. In contrast, knockdown of SNHG22 or E2F3 significantly weakened the proliferation, migratory, and invasive capacity of Lovo cells; nevertheless, these inhibitory effects could be attenuated by co-transfection with miR-128-3p inhibitors (Fig. 7A-F). These results showed that miR-128-3p/E2F3 reversed the stimulating effects of SNHG22 on the proliferative, migratory, and invasive capacity of CRC cells.

Discussion

Accumulating evidence has recently revealed that dysregulation of lncRNAs plays an essential role in the development and invasion of diverse human cancers, including CRC.^{17, 18} Here, we showed that SNHG22 expression was significantly elevated in CRC tissues and cell lines. High levels of SNHG22 expression were significantly correlated with unfavorable clinicopathological characteristics and worse survival in patients with CRC. Functionally, ectopic SNHG22 could drive cell proliferation, migration, invasion, and prohibit cell apoptosis in CRC cell lines. Knockdown of SNHG22 prohibited xenograft tumor growth in vivo. We further confirmed that SNHG22 executed tumor-promoting function through sponging miR-128-3p and enhancing the expression and activity of E2F3. To the best of our knowledge, our results are the first to clarify that SNHG22 acts as an oncogene in CRC, and can be used as a potential therapeutic target for this disease.

The current anatomic-based TNM staging system is not able to precisely distinguish the risk of recurrence/distant metastasis in CRC patients. So, it is essential to find the novel prognostic biomarkers. Dysregulation of lncRNAs in various types of the human tumor was associated with excellent or dismal survival, making them promising prognostic biomarkers.^{19, 20} For example, SNHG7 expression is enhanced in CRC tissues as compared with noncancerous tissues, and this high expression is related to aggressive pathological characteristics, such as TNM stage, lymphatic metastasis, and distant metastasis, as well as poor prognosis.^{8, 9} Upregulation of PVT1 was an indicator of dismal survival in

several cancers, including CRC.²⁰ Some researchers have reported the upregulated expression of SNHG22 in EOC, PTC, and ccRCC, and whose expression indicated poor prognosis in the three types of human malignancies.^{11–13} In this study, we first explored the expression patterns of SNHG22 in ANT, CRA, and CRC tissues, and found that SNHG22 expression was gradually upregulated in ANTs, CRA, and TTs. The high expression level of SNHG22 was associated with advanced T stage, node involvement, metastasis, and poor survival in CRC patients. So, SNHG22 may serve as an independent prognostic indicator in patients with CRC.

Reports have found that being as oncogenes, several SNHG family members may play critical roles in the processes of tumor advancement. Initially, SNHG22 was shown to be highly expressed in EOC, and could stimulate EOC cell proliferation, invasion, and chemotherapy resistance in EOC.¹¹ SNHG22 was then determined to be highly expressed in PTC and ccRCC.^{12, 13} We also checked the effects of SNHG22 on cell phenotype of CRC. We did prove that SNHG22 could promote cell proliferation, invasion, migration, and inhibit cell apoptosis of CRC in vitro. Knockdown of SNHG22 in vivo prohibited xenograft tumor growth. All these results in this report implicate that SNHG22 may act as a critical oncogenic lncRNA in CRC.

Most cytoplasmic lncRNAs exert their effects on the regulation of gene expression through function as ceRNAs, in which lncRNAs regulate miRNAs by competitively binding their target sites on protein-coding mRNA molecules.^{21, 22} For example, Acting as a ceRNA, MIR17HG bases its biological behaviors on a sequence-specific interaction with miRNA-17-5p, thus attenuating their roles in mRNA targets by titrating the amount of miRNA.⁵ Chen et al. found that lncRNA UICLM promoted the invasion of CRC cell lines by sponging miR-215.²⁹ Based on computer analyses and experimental assays, we demonstrated that SNHG22 was preferentially localized to the cytoplasm. Furthermore, we searched and confirmed the miRNAs that SNHG22 could sponge in CRC, and the dual-luciferase reporter assay indicated that SNHG22 could interact with miR-128-3p and hinder its biological roles. Previous studies have reported that miR-128-3p could function as a tumor suppressor in several types of human cancer,^{23–27} involving in cell proliferation, cell cycle, and chemosensitivity. Concerning CRC, it is suggested that the miR-128-3p enhance the chemosensitivity of oxaliplatin-resistant CRC cells by targeting Bmi1 and MRP5.²⁵ In addition, the nanocomplexes loaded with miR-128-3p could elevate chemotherapy roles through dual-targeting silence the activity of PI3K/AKT and MEK/ERK pathway in CRC cell lines.²⁴ We also confirmed the anti-carcinogenic effects of miR-128-3p on CRC cell lines in this study, and the function of miR-128-3p is inhibited after sponging by SNHG22.

As a ceRNA, the function of lncRNA depends on the miRNA target gene. Using five online databases, we predicted E2F3 as a potential target of miR-128-3p. Recent studies have uncovered that E2F3 plays an essential role in the regulation of cell proliferation, apoptosis, and chemoresistance of human cancer cells.^{28, 29} Initially, we performed luciferase reporter assays to validate this hypothesis. Furthermore, overexpression of miR-128-3p inhibited, while silencing of miR-128-3p elevated, the mRNA and protein

expression levels of E2F3. Further functional rescue experiments validated that SNHG22 regulates CRC proliferation and invasion by competitively sponging miR-128-3p and restoring the activity of E2F3.

In summary, our results revealed that SNHG22, as an oncogenic lncRNA in CRC, is upregulated and related to the dismal survival of CRC patients. Functional and mechanistic analyses show that SNHG22 promotes CRC tumorigenesis and metastasis via sponging miR-128-3p, leading to elevated expression of E2F3. These findings may provide new insights into the development of novel therapeutics for CRC.

Abbreviation

CRC, colorectal cancer; EOC, epithelial ovarian carcinoma; PTC, papillary thyroid cancer; ccRCC, clear cell renal cell carcinoma; lncRNA, long non-coding RNA; snoRNA, small nucleolar RNA; SNHG, small nucleolar RNA host gene; siRNA, small interfering RNA; Lv, lentivirus; UTR, untranslated region; mut, mutant-type; RIP, RNA immunoprecipitation; CCK-8, Cell Counting Kit-8; IHC, immunohistochemistry; TT, tumor tissue; ANT, adjacent nontumor tissue; TCGA, the cancer genome atlas; DFS, disease-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; ceRNA, competing endogenous RNA.

Declarations

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University. The research has been carried out in accordance with the World Medical Association Declaration of Helsinki.

Author Contributions:

Conception and design: YJN, WCF, DXY.

Acquisition of data: YJN, LYL, ZHN, ZDY.

Analysis and interpretation of data: YJN, WCF, DXY, ZYZ.

Writing, review, and/or revision of the manuscript: YJN, WCF.

Study supervision: YJN.

Final approval of manuscript: All authors.

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Availability of data and materials

The raw data used and analyzed in the current study are available from the corresponding author upon a reasonable request.

Competing interests:

The authors disclose no conflict of interest.

Consent for publication

All patients provided written informed consent prior to their inclusion within the study.

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References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018; 68: 394-424.
2. De Angelis R, Sant M, Coleman MP, Francisci S, Baili P, Pierannunzio D et al. Cancer survival in Europe 1999-2007 by country and age: results of EURO CARE-5-a population-based study. *Lancet Oncol* 2014; 15: 23-34.
3. He Q, Long J, Yin Y, Li Y, Lei X, Li Z et al. Emerging Roles of lncRNAs in the Formation and Progression of Colorectal Cancer. *Front Oncol* 2019; 9: 1542.
4. Luo Y, Yang J, Yu J, Liu X, Yu C, Hu J et al. Long Non-coding RNAs: Emerging Roles in the Immunosuppressive Tumor Microenvironment. *Front Oncol* 2020; 10: 48.
5. Xu J, Meng Q, Li X, Yang H, Xu J, Gao N et al. Long Noncoding RNA MIR17HG Promotes Colorectal Cancer Progression via miR-17-5p. *Cancer Res* 2019; 79: 4882-95.
6. Ding D, Li C, Zhao T, Li D, Yang L, Zhang B. LncRNA H19/miR-29b-3p/PGRN Axis Promoted Epithelial-Mesenchymal Transition of Colorectal Cancer Cells by Acting on Wnt Signaling. *Mol Cells* 2018; 41: 423-35.
7. Williams GT, Farzaneh F. Are snoRNAs and snoRNA host genes new players in cancer? *Nat Rev Cancer* 2012; 12: 84-8.
8. Shan Y, Ma J, Pan Y, Hu J, Liu B, Jia L. LncRNA SNHG7 sponges miR-216b to promote proliferation and liver metastasis of colorectal cancer through upregulating GALNT1. *Cell Death Dis* 2018; 9: 722.
9. Li Y, Zeng C, Hu J, Pan Y, Shan Y, Liu B et al. Long non-coding RNA-SNHG7 acts as a target of miR-34a to increase GALNT7 level and regulate PI3K/Akt/mTOR pathway in colorectal cancer progression. *J Hematol Oncol* 2018; 11: 89.
10. Ota T, Suzuki Y, Nishikawa T, Otsuki T, Sugiyama T, Irie R et al. Complete sequencing and characterization of 21,243 full-length human cDNAs. *Nat Genet* 2004; 36: 40-5.

11. Zhang PF, Wu J, Luo JH, Li KS, Wang F, Huang W et al. SNHG22 overexpression indicates poor prognosis and induces chemotherapy resistance via the miR-2467/Gal-1 signaling pathway in epithelial ovarian carcinoma. *Aging (Albany NY)* 2019; 11: 8204-16.
12. Yang W, Zhang K, Li L, Ma K, Hong B, Gong Y et al. Discovery and validation of the prognostic value of the lncRNAs encoding snoRNAs in patients with clear cell renal cell carcinoma. *Aging (Albany NY)* 2020; 12: 4424-44.
13. Gao H, Sun X, Wang H, Zheng Y. Long non-coding RNA SNHG22 increases ZEB1 expression via competitive binding with microRNA-429 to promote the malignant development of papillary thyroid cancer. *Cell Cycle* 2020; 19: 1186-99.
14. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; 25: 402-8.
15. Bachmann IM, Halvorsen OJ, Collett K, Stefansson IM, Straume O, Haukaas SA et al. EZH2 expression is associated with high proliferation rate and aggressive tumor subgroups in cutaneous melanoma and cancers of the endometrium, prostate, and breast. *J Clin Oncol* 2006; 24: 268-73.
16. Cao Z, Pan X, Yang Y, Huang Y, Shen HB. The lncLocator: a subcellular localization predictor for long non-coding RNAs based on a stacked ensemble classifier. *Bioinformatics* 2018; 34: 2185-94.
17. Lan Y, Xiao X, He Z, Luo Y, Wu C, Li L et al. Long non-coding RNA OCC-1 suppresses cell growth through destabilizing HuR protein in colorectal cancer. *Nucleic Acids Res* 2018; 46: 5809-21.
18. Wang Y, Lu JH, Wu QN, Jin Y, Wang DS, Chen YX et al. lncRNA LINRIS stabilizes IGF2BP2 and promotes the aerobic glycolysis in colorectal cancer. *Mol Cancer* 2019; 18: 174.
19. Wang D, Zhang H, Fang X, Zhang X, Liu H. Prognostic value of long non-coding RNA GHET1 in cancers: a systematic review and meta-analysis. *Cancer Cell Int* 2020; 20: 109.
20. Xiao M, Feng Y, Liu C, Zhang Z. Prognostic values of long non-coding RNA PVT1 in various carcinomas: An updated systematic review and meta-analysis. *Cell Prolif* 2018; 51: e12519.
21. Li M, Bian Z, Jin G, Zhang J, Yao S, Feng Y et al. lncRNA-SNHG15 enhances cell proliferation in colorectal cancer by inhibiting miR-338-3p. *Cancer Med* 2019; 8: 2404-13.
22. Yan J, Jia Y, Chen H, Chen W, Zhou X. Long non-coding RNA PXN-AS1 suppresses pancreatic cancer progression by acting as a competing endogenous RNA of miR-3064 to upregulate PIP4K2B expression. *J Exp Clin Cancer Res* 2019; 38: 390.
23. Zhao J, Li D, Fang L. MiR-128-3p suppresses breast cancer cellular progression via targeting LIMK1. *Biomed Pharmacother* 2019; 115: 108947.
24. Liu X, Dong C, Ma S, Wang Y, Lin T, Li Y et al. Nanocomplexes loaded with miR-128-3p for enhancing chemotherapy effect of colorectal cancer through dual-targeting silence the activity of PI3K/AKT and MEK/ERK pathway. *Drug Deliv* 2020; 27: 323-33.
25. Liu T, Zhang X, Du L, Wang Y, Liu X, Tian H et al. Exosome-transmitted miR-128-3p increase chemosensitivity of oxaliplatin-resistant colorectal cancer. *Mol Cancer* 2019; 18: 43.

26. Li X, Lv X, Li Z, Li C, Li X, Xiao J et al. Long Noncoding RNA ASLNC07322 Functions in VEGF-C Expression Regulated by Smad4 during Colon Cancer Metastasis. *Mol Ther Nucleic Acids* 2019; 18: 851-62.
27. Fu C, Li D, Zhang X, Liu N, Chi G, Jin X. LncRNA PVT1 Facilitates Tumorigenesis and Progression of Glioma via Regulation of MiR-128-3p/GREM1 Axis and BMP Signaling Pathway. *Neurotherapeutics* 2018; 15: 1139-57.
28. Wang H, Wang L, Zhang S, Xu Z, Zhang G. Downregulation of LINC00665 confers decreased cell proliferation and invasion via the miR-138-5p/E2F3 signaling pathway in NSCLC. *Biomed Pharmacother* 2020; 127: 110214.
29. Chen J, Liu X, Xu Y, Zhang K, Huang J, Pan B et al. TFAP2C-Activated MALAT1 Modulates the Chemoresistance of Docetaxel-Resistant Lung Adenocarcinoma Cells. *Mol Ther Nucleic Acids* 2019; 14: 567-82.

Figures

Figure 1

The differential expression of SNHG22 in CRC tissues and cell lines. A, qRT-PCR assay analysis of the expression levels of SNHG22 in TTs (n = 93), ANT (n = 93) and adenoma (n=33) colon tissues. B-E, The differential expression of SNHG22 in CRC tissues was analyzed according to T stage (B), node status (C), distant metastasis (D) and TNM stage (E). F-G, Kaplan-Meier survival analysis of the SNHG22 level and DFS (F) and OS (G) of CRC patients from our cohort. H-I, expression of SNHG22 in CRC patients and TNM stage and OS based on TCGA-COAD dataset. J, qRT-PCR assay analysis of the expression levels of SNHG22 in CRC cell lines and FHC cell line, a human normal colon epithelial cell line. CRC, colorectal cancer; TT, tumor tissue; ANT, adjacent noncancerous tissues. Data are presented as means $\pm$ SD from triplicate experiments. *P < 0.05; **P < 0.01; ***P < 0.001.

Figure 2

Overexpression of SNHG22 promoted CRC cells proliferation, migration and invasion, and inhibited apoptosis in vitro. A-B, CCK-8 assay analysis of the proliferative ability of Caco2 and LS147T cells transfected with the indicated vectors. C-F, Flow cytometry assay analysis of the apoptotic rates and cell cycle progression of Caco2 and LS147T cells transfected with the indicated vectors. G-H, Wound scratch assay analysis of the migration of Caco2 and LS147T cells transfected with the indicated vectors. I-J,

Transwell assay analysis of the migration and invasion of Caco2 and LS147T cells transfected with the indicated vectors. CRC, colorectal cancer; NC, negative control; n.s., not significant. Data are presented as means $\pm$ SD from triplicate experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ relative to the control.

Figure 3

Knockdown of SNHG22 inhibited cell proliferation, migration and invasion, but promoted apoptosis of CRC cells in vitro. A, CCK-8 assay analysis of the proliferative ability of LoVo cells after transfection with the indicated vectors. B-C, Flow cytometry assay analysis of the apoptotic rates and cell cycle progression of LoVo cells after transfection with the indicated vectors. D, Wound scratch assay analysis of the migration of LoVo cells after transfection with the indicated vectors. E, Transwell invasion assay analysis of the invasion of LoVo cells after transfection with the indicated vectors. CRC, colorectal cancer;

NC, negative control; n.s., not significant. Data are presented as means $\pm$ SD from triplicate experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ relative to the control.

Figure 4

Knockdown of SNHG22 inhibits tumor xenograft growth of CRC in vivo A, LoVo cells were inoculated in BALB/c nude mice (n=5 for each group) to establish subcutaneous xenograft tumors, and the dissected tumors were photographed. Scale bar, 10mm. B-C, Effects of SNHG22 knockdown in LoVo cells on tumor volumes (B) and tumor weights (C) in the subcutaneous xenografts mice models. D, qRT-PCR assay analysis of expression levels of SNHG22 in the subcutaneous xenografts mice models. E-F, IHC assay analysis of the expression levels of Ki-67 staining in the subcutaneous xenografts mice models. Scale bar, 100 μ M. Data are presented as means $\pm$ SD from triplicate experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ relative to the control.

Figure 5

SNHG22 functions as a ceRNA to regulate miR-128-3p expression in CRC A, Subcellular localization of SNHG22 in CRC cell lines (Caco2, LS147T and LoVo). Actin and U6 served as a cytoplasmic and nuclear localization marker, respectively. B, The predicted binding sites of miR-128-3p to the SNHG22 sequence. C, RIP assay was performed with an antibody against Ago2 in Caco2 and LS147T cell lines transfected with NC mimic and miR-128-3p mimic. D, Luciferase activity of 293T, Caco2 and LS147T cell lines co-transfected with miR-128-3p mimic (or NC mimic) and luciferase reporters containing SNHG22 wt or SNHG22 mut transcript were analyzed. E-F, qRT-PCR assay analysis of the expression levels of miR-128-3p in CRC cells after transfection with the indicated vectors. G, A negative correlation between SNHG22 and miR-128-3p expression in human CRC tissues from the Zhengzhou cohort (Spearman's correlation analysis, $R = -0.3857$, $P < 0.001$). RIP, RNA immunoprecipitation; Ago2, Argonaute2; CRC, colorectal cancer; NC, negative control; wt, wild-type; mut, mutant-type; n.s., not significant. Data are presented as means $\pm$ SD from triplicate experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ relative to the control.

Figure 6

MiR-128-3p targets and regulates E2F3 A, A Venn diagram showed the number of genes identified as potential targets of miR-128-3p. B, TCGA-COAD dataset showed the E2F3 expression levels in TTs and ANT. C-F, qRT-PCR and western blot assays analysis of expression levels of E2F3 mRNA and protein in Caco2 and LS147T cells after transfection with the indicated vectors. G, Significantly inverse correlations between miR-128-3p and E2F3 mRNA in human CRC tissues from the Zhengzhou cohort ($R = -0.2487$, $P = 0.016$). H, The miR-128-3p putative binding sites and corresponding mutant sequence of E2F3. I, Dual-luciferase reporter assays in CRC cell lines co-transfected with miR-128-3p mimic and luciferase reporters

containing wt or mut E2F3 transcript. The relative luciferase activity was normalized to the Renilla luciferase activity. J, A positive correlation between SNHG22 and E2F3 expression in human CRC tissues from Zhengzhou cohort ($R = -0.4047$, $P < 0.001$). COAD, colon adenocarcinoma; CRC, colorectal cancer; NC, negative control; wt, wild-type; mut, mutant-type; n.s., not significant. Data are presented as means $\pm$ SD from triplicate experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ relative to the control.

Figure 7

SNHG22 exerts its function by inhibiting the miR-128-3p/E2F3 axis in CRC cells A-B, CCK-8 assay analysis of the proliferative ability of Caco2 and LoVo cells after transfection with the indicated vectors. C-D, Wound scratch assay analysis of the migratory ability of Caco2 and LoVo cells after transfection with the indicated vectors. E-F, Transwell assay analysis of the invasive ability of Caco2 and LoVo cells after transfection with the indicated vectors. Data are presented as means $\pm$ SD from triplicate experiments. *P < 0.05; **P < 0.01; ***P<0.001 relative to the control.

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