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Original Paper

SNHG6 Promotes Tumor Growth via Repression of P21 in Colorectal Cancer

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Key Words
SNHG6 • P21 • Colorectal cancer • Cell proliferation

Abstract
Background/Aims: SNHG6 (Small Nucleolar RNA Host Gene 6) is a novel non-coding RNA (ncRNA) and its cellular function is largely unknown. ***Methods:*** Cell Counting Kit-8 (CCK-8) cell growth assay, colony formation and flow cytometry were used to determine colorectal cancer cell proliferation, cell cycle progression and apoptosis *in vitro*. The xenograft tumor formation assay in nude mice was established to evaluate tumor growth *in vivo*. RNA immunopreciptation (RIP) analysis was performed to examine whether SNHG6 could bind to EZH2 (enhancer of zeste 2 polycomb repressive complex 2 subunit), and chromatin immunoprecipitation (ChIP) assay was conducted to examine whether SNHG6 could repress p21 transcription by recruiting EZH2 to the p21 promoter. ***Results:*** Here we found that SNHG6 was upregulated and its expression levels were positively correlated with advanced tumor stage in colorectal cancer. Survival analysis suggested that higher expression of SNHG6 predicted poor prognosis in patients with colorectal cancer. Functional studies indicated that SNHG6 could promote cell proliferation via a direct suppression of p21 expression in colorectal cancer cells. Moreover, SNHG6 repressed p21 transcription through recruiting EZH2 to the p21 promoter in colorectal cancer cells. ***Conclusion:*** Taken together, our study demonstrates that SNHG6 promotes tumor growth via repression of p21 in colorectal cancer, which may provide a promising target for novel anticancer therapeutics.

Introduction

Colorectal cancer (CRC) is one of the most frequent malignancies in the world and the leading cause of cancer-related mortality [1, 2]. CRC is a highly heterogeneous disease with diverse genetic and clinical manifestations which can have significant impacts on therapeutic outcomes [3-5]. Still, the prognosis of colorectal cancer patients is poor due to the cancer recurrence and distant metastasis despite treatment advances over the past decades [1, 3,

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6]. Until now the molecular mechanisms involved in colorectal cancer tumorigenesis and progression remain largely unknown.

With the advancement of genome sequencing and analysis, tens of thousands of RNA transcripts lacking protein-coding potential have been discovered in the past decade [7]. RNAs without coding potential are referred to as non-coding RNAs (ncRNAs)[8]. Although most ncRNAs were considered to be evolutionary junk in the past, increasing evidence suggests that ncRNAs may act as key regulators of diverse biological and disease processes [9]. Small nucleolar RNAs (snoRNAs) are a subset of ncRNA molecules which regulate various cellular processes including RNA modification, processing and alternative splicing [10-12]. SNHG6 is a novel snoRNA host gene that plays a vital role in tumor biology, its deregulated expression has been found in several cancers. However, the role of SNHG6 in the tumorigenesis of colorectal cancer is not clear.

The cyclin-dependent kinase inhibitor p21 (encoded by CDKN1A) belongs to the Cip/Kip family of CDK inhibitors, which includes p21, p27 and p57[13]. p21 could prevent cell cycle progression by inhibiting CDK2 activity, which is required for the phosphorylation of Rb and subsequent E2F-dependent gene expression [14]. Moreover, p21 is a major target of p53 which is associated with linking DNA damage to cell cycle arrest [15]. Given that the ability of p21 to inhibit cell proliferation may contribute to its tumor suppressor function, it is not surprising that various oncogenes repress p21 to promote cell growth and tumorigenesis [16, 17]. Notably, loss of expression or function of p21 has been implicated in the genesis or progression of many human cancers [18, 19]. Also, growing evidence suggests that functional loss of p21 could mediate a drug resistance phenotype [18, 19].

In our study, we found that SNHG6 was overexpressed in colorectal cancers, and its expression levels were positively correlated with advanced colorectal cancer stage. Functional studies showed that SNHG6 could promote cell proliferation via a direct suppression of p21 expression in colorectal cancer. What's more, survival analysis suggested that increased SNHG6 expression associated with poor prognosis and worse cancer-specific survival in colorectal cancer.

Materials and Methods

Cell culture

SW480 and HT-29 cells (Cell bank of Chinese Academy of Sciences, Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), 0.1 mg/ml streptomycin, and 100 units/ml penicillin (Invitrogen, California, USA) in 5% CO_2 atmosphere at 37°C.

Stable cell lines

Establishment of stable cell lines was performed as previously described [20]. Briefly, lentiviral plasmids expressing SNHG-shRNAs or p21-shRNAs were co-transfected with pHelper plasmids in 293T cells. Lentiviral particles were harvested and purified with ultracentrifugation from the media after 48 hours of transfection. Cells were then infected with lentiviruses encoding SNHG-shRNAs or p21-shRNAs. The efficiency of knockdown was evaluated by real-time PCR or western blot analysis.

Cell Counting Kit-8 assay

Cell proliferation was evaluated using Cell Counting Kit-8 (CCK-8, Dojindo, Tokyo, Japan) according to the manufacturer's instructions. The absorbance value for each well was measured at 450 nm with a Multiskan FC microplate reader (Thermo scientific, Waltham, MA, USA). The experiment was conducted three times.

Colony formation assay

Cells (2.0×10^3) were seeded into 6-well plates in triplicate in 2 ml of complete growth medium. The medium was changed every three days. Two weeks later, cells were stained by 0.1% crystal violet (Sigma-Aldrich, St. Louis, MO, USA) in methanol for 10 min. Colonies (more than 50μm diameter) were counted directly on the plate.

BrdU (Bromodeoxyuridine) incorporation

Cells were exposed to 10 μM BrdU (BD Biosciences, Mountain View, CA) for 30 min and fixed in 70% ethanol, and then washed with PBS, resuspended, and incubated with 4N HCL and 0.5% Triton X-100 for 30 min at room temperature. After washing with PBS, cells were neutralized with 0.1 M sodium borate before being labeled with FITC-conjugated BrdU antibody (BD Biosciences, Mountain View, CA) and incubated with 50 μg/ml propidium iodide (Sigma Chemical Company, St. Louis, MO) according to the manufacturer's protocol before being analyzed by a Becton Dickinson FACStar Plus flow cytometer.

Quantitative real-time PCR

Following the 'Minimal Information for Publication of Quantitative Real-Time PCR Experiments' (MIQE) guidelines [21], quantitative RT-PCR was performed using SYBR Green dye on an Applied Biosystems 7300 Real-time PCR system (Applied Biosystems, Foster City, CA). (1) Experimental design and sample preparation: Tumor tissues of colorectal cancer (n=66) and non-tumor colon tissues (n=15) were obtained from the First Affiliated Hospital of Zhengzhou University. All samples were immediately frozen in liquid nitrogen after they were washed with pre-cold PBS, and then were stored at –80°C until RNA extraction. (2) Nucleic acid extraction: Total RNAs were extracted from tumor tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, 1 ml of Trizol Reagent was added to 50 mg of tissue. Vortexed the homogenized samples well and then incubated for 5 min at room temperature to completely dissociate nucleoprotein complexes. After homogenization, phase separation was performed with 200μl of chloroform (Merck, New Jersey, USA) at 12000g for 15min. Then carefully transferred the aqueous phase to a fresh tube, avoiding the interphase altogether. After supplying 500μl of isopropyl alcohol (Thermo Fisher Scientific, Waltham, MA, USA), the mixture was allowed to incubate at room temperature for 10 min. Following centrifugation at 12000g for 10 min total RNA was precipitated from the aqueous phase. Continue with the manufacturer's protocol as described. After washing once with 1ml of 75% ethanol, centrifugation was done at 7500g for 5 min at 4°C. Finally, RNA pellet was resuspended in 40μl RNase-free water (Thermo Fisher Scientific, Waltham, MA, USA) and stored at –80°C. For the DNase treatment, 1ug of RNA was treated with 1μl of gDNA Erase and 2μl gDNA Eraser Buffer (Thermo Fisher Scientific, Waltham, MA, USA) in a 20ul final volume reaction. Digestion of DNA was achieved with 2 minutes incubation at 42 °C. Reverse transcription controls (without enzyme) were performed in order to assess the existence of DNA in the RNA sample. For that purpose, RNA was processed as a normal sample in the RT step, except that no reverse transcriptase was added to the reaction mixture. RNA concentration was assessed using spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) by measuring the absorbance at 260 nm UV light. RNA purity was determined by measuring the absorbance at OD260 and OD280. RNA integrity was assessed using Agilent's 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). (3) Reverse transcription was performed using SuperScript ® III First-Strand Synthesis System (#18080-051). The complete reaction conditions are followings: 1μl Primer (50μM oligo dT) and 1μl 10mM dNTP mix were added to each mRNA sample. The cDNA synthesis master mix contained (per reaction): 2μl 10x RT buffer, 4μl 25mM MgCl2, 2μl 0.1M DTT, 1μl RNaseOUT (40U/μl), 1μl Superscript III RT (200U/μl). 1μl RNase H was added to each reaction following termination of cDNA synthesis to degrade any RNA template, with the Superscript® III reverse transcriptase (Invitrogen) at a concentration of 200U/μl, 50pmol Oligo dTprimer in each 20μl reaction system. The Reverse transcription reaction conditions are as following: first, samples were incubated for 5 minutes at 65°C and placed on ice for 1 minute. Following addition of the mastermix, samples were incubated for 50 minutes at 50°C. The reactions were terminated following incubation at 85°C for 5 minutes. Following addition of RNase H (Invitrogen), samples were incubated at 37°C for 20 minutes. (4) Primers for SNHG6 (NR_002599) and GAPDH (NM_002046) were purchased from the Sangon biotechnology (Shanghai, China). SNHG6 (102bp): F-ATACTTCTGCTTCGTTACCT; R-CTCATTTTCATCATTTGCT; GAPDH(158bp): F-GGTATCGTGGAAGGACTCA; R-CCTTGCCCACAGCCTTG.; (5) qPCR was performed using SYBR® Premix Ex TagTM II (TakaRa) in 20μl

reaction volumes. In brief, each reaction was comprised of 2μl of the cDNA solution, 10μl of SYBR® Premix Ex TagTM (2×), 1.6μl of primers, 0.4μl of ROX Reference Dye II and 6μl of nuclease-free water. All qPCR reactions were performed on ABI 7300 Real-Time PCR System (Applied Biosystems, USA). (6) Gene-specific amplification was confirmed by a single band in 2% agarose gel electrophoresis stained with ethidium bromide. PCR efficiency of SNHG6 and GAPDH are 99.4% and 98.8%, respectively. (7) Data were analyzed by Quantification Software version 2.1 (Applied Biosystems, USA). The threshold was defined as the fractional cycle number at which the fluorescence exceeded the given threshold and was calculated using SDS Relative Quantification Software version 2.1 using the automatic baseline setting. The experiments were performed independently three times. Comparisons between groups were performed with Student's t-test statistical analysis was carried out using IBM SPSS Statistics 19 software (IBM Corp., Armonk, NY, USA).

Western blot analysis

Western blot analysis was performed as previously described [22]. Briefly, cells were lysed in cold lysis buffer, proteins (20-30μg) were resolved on SDS-PAGE, transferred onto PVDF membranes, and probed with antibodies to p21 (10355-1-AP, Proteintech), EZH2 (21800-1-AP, Proteintech), H3K27me (ab192985, Abcam) and GAPDH (sc-32233, Santa Cruz Biotechnology) at 4°C overnight. Detection was performed with the SuperSignal West Femto Maximum Sensitivity Substrate Trial Kit (Pierce, Rockford, IL, USA). Detection was carried out with the SuperSignal West Femto Maximum Sensitivity Substrate Trial Kit (Pierce, Rockford, IL, USA). The band images were digitally captured and quantified with a FluorChem FC2 imaging system (Alpha Innotech, San Leandro, CA, USA).

Immunohistochemistry

This study was approved by the Research Ethics Committee of Zhengzhou University. Written informed consents were obtained from all patients who provided samples. Colorectal cancer samples were collected at the time of diagnosis from the First Affiliated Hospital of Zhengzhou University. Immunohistochemistry analysis was performed as previously described. The relative protein expression was evaluated by the average percentage of positive cells (number of positive cells ×100/total number of cells) in 5 different random microscopic fields in each tumor sample.

RNA Immunoprecipitation (RIP) Assay

RIP experiments were performed using the Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA) following the manufacturer's instructions. Antibodies for RIP-assay were EZH2 (21800-1-AP, Proteintech) or normal IgG, (ab199376, Abcam).

Chromatin Immunoprecipitation (ChIP) Assay

The ChIP assays were performed as previously reported [23]. The ChIP assay kit (Upstate Biotechnology, Inc., Lake Placid, NY) was used as recommended by the manufacturer. Briefly, approximately 1.0×10^7 cells were fixed with 1% formaldehyde for 10 min at room temperature, followed by washing with 20ml of cold 1×PBS twice and harvested by scraping. Cells were then lysed in 1 mL of SDS Lysis Buffer containing Protease Inhibitor Cocktail II. The cell lysate was sonicated on wet ice four times for 15 seconds each time with 15-second intervals to obtain chromatin fragments of about 200–1000 bp nucleotides. Insoluble materials were removed by centrifugation at 12, 000×g at 4°C for 10 min. Each 100 μL supernatants were diluted with 900 μL of ChIP dilution buffer and preincubated with Protein G agarose at 4°C for 1 hour, and then pelleted agarose by brief centrifugation and removed 10 μL (1%) of the supernatant as Input. Supernatants were incubated at 4°C overnight with 5 μg of EZH2 (21800-1-AP, Proteintech), H3K27me (ab192985, Abcam) or normal IgG, (ab199376, Abcam) incubated overnight at 4°C with rotation, and then added 60 μL of Protein G Agarose to each tube and incubated for 1 hour at 4°C with rotation. After washing with a washing buffer, the immunoprecipitates were eluted and reverse cross-linked by incubation overnight at 65°C in elution buffer. DNA was then purified with a PCR purification kit (Qiagen, Hilden, Germany). Immunoprecipitated DNAs were analyzed by quantitative real-time PCR.

Xenograft tumor formation

Xenograft tumor formation assay were performed as previously described [20]. Briefly, the BALB/c (6–8 weeks old) athymic nude mice were purchased from Vital River Laboratory Animal Technology (Charles River Laboratories, Beijing, China). The mice were subcutaneously injected in the flank regions with 1.0×10^6 cells in 0.1 mL of PBS. The tumor size was measured twice a week with calipers. The tumor volume was calculated with the formula: (Length × Width^2)/2. Four weeks after implantation, mice were euthanized by asphyxiation in a CO_2 chamber and tumors were excised and examined. All procedures were conducted in accordance to Animal Care and Use Committee guidelines of Zhengzhou University.

Analysis of microarray data

Oncomine cancer microarray database (http://www.oncomine.org) was used to study the gene expression of SNHG6 in colorectal cancer samples. Gene expression data were also obtained from NCBI Gene Expression Omnibus (GEO) database (accession numbers: GSE8671, GSE9348, GSE5206, GSE9689 and GSE20916), and SNHG6 expression data were log transformed, median centered per array, and the standard deviation was normalized to one per array. The co-expression analysis of SNHG6 and p21 in five colorectal cancer datasets (TCGA, GSE14333, GSE37892, GSE20916 and GSE18105) was analyzed through querying the open database ChIPBase v2.0 and the R2 Genomics Analysis and Visualization Platform (http://r2.amc.nl). The univariate survival analysis within the colorectal cancer data set of the GSE16011 (n=226) was performed using the Kaplan-Meier analysis module of the R2 microarray analysis and visualization platform.

Statistical analysis

All data were expressed as mean ± standard error of the mean (SEM). Between groups and among groups comparisons were conducted with Student t test and ANOVA, respectively. Mann-Whitney U test is used for nonparametric variables. The association analysis of SNHG6 expression and clinical characteristics was estimated by Chi-square or Fisher's two-tailed exact test. Statistical analysis was performed using GraphPad Prism software version 4.0 (PRISM4) (GraphPad Software Inc, LaJolla, CA), and $p<0.05$ was considered significant.

Results

SNHG6 is overexpressed in colorectal cancers and higher SNHG6 expression predicts a worse progression-free survival

To address the role of SNHG6 in colorectal cancer development, we examined the expression of SNHG6 in colorectal cancer. First, we evaluated the expression level of SNHG6 mRNA in colorectal cancers by querying the public available ONCOMINE database (www.oncomine.org) [24]. Five colorectal cancer microarray expression datasets were analyzed. The results indicated that the expression of SNHG6 mRNA is significantly higher in colorectal cancer than that in non-tumor colorectal tissues in these microarray studies. Fold increase of the levels of SNHG6 mRNA in GSE8671, GSE9348, GSE5206, GSE9689 and GSE20916 were 1.97, 3.06, 2.14, 1.91 and 2.42, respectively (Fig. 1A-1E). Next, the expression of SNHG6 in 66 colorectal cancer tissues and 15 non-tumor colon tissues were examined by real-time PCR. The results showed that SNHG6 was overexpressed in our own colorectal cancer patient cohort (Fig. 1F), which was consistent with the previous microarray gene expression studies. Moreover, the correlation between the expression pattern of SNHG6 and the clinicopathological characteristics of colorectal cancer were analyzed (Table 1). We found no significant association between SNHG6 and age, gender, tumor location, tumor grade, distant metastases and tumor recurrence, except for tumor stage (Table 1). In our study, overexpression of SNHG6 was detected in 18 out of 25 patients with high-stage (III/IV) colorectal cancers ($p=0.011$; Table 1).

Furthermore, we explored the relationship between SNHG6 expression and prognosis in subjects with colorectal cancer. In our patient cohort, the univariate analysis showed that patients with higher SNHG6 expression (n=33) had much worse progression-free survival

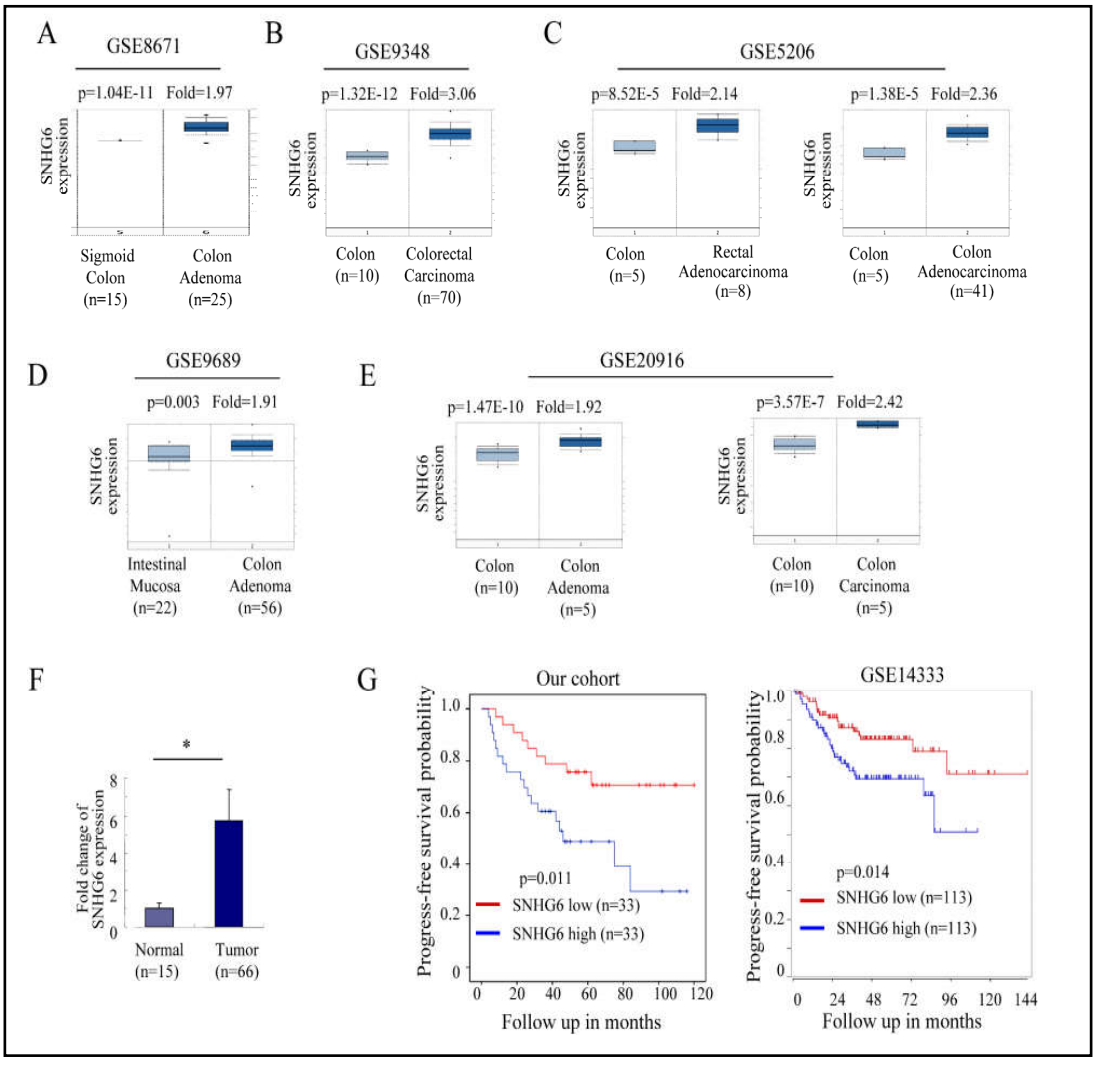

Fig. 1. SNHG6 is overexpressed in colorectal cancer and higher expression of SNHG6 predicts a worse progress-free survival. A-E. The expression of SNHG6 is upregulated in colorectal cancer tissues when compared with non-tumor colorectal tissues. All data, including fold change and p-values, were calculated from ONCOMINE (www.oncomine.org). F. The expression of SNHG6 in 66 colorectal cancer tissues and 15 non-tumor colon tissues were examined by real-time PCR. Bars, s.e.m.; *, p<0.05. G. Univariate analysis of progress-free survival was performed using the Kaplan-Meier method analysis in our colorectal cancer patient cohort (n=66; p=0.011) and GSE14333 dataset (n=226, p=0.014).

than those with lower level of SNHG6 (n=33). The estimated five-year disease progression-free survival rates for those of the higher and lower expression groups were 49.0% and 75.8%, respectively (P=0.011; Fig. 1G). The public available colorectal cancer dataset (GSE14333) was used to conduct survival analysis. This analysis included 226 patients with colorectal cancer. The univariate analysis of survival was performed using the Kaplan-Meier analysis module of the R2 microarray analysis and visualization platform (http://r2.amc.nl). Patients with colorectal cancer were divided into SNHG6 low expression group (SNHG6 low; n=113) and SNHG6 high expression group (SNHG6 high; n=113). The Kaplan–Meier analysis indicated that colorectal cancer patients with high expression of SNHG6 had worse progression-free survival than those with lower expression of SNHG6 (p=0.014; Fig. 1G). Collectively, these data indicated that patients with higher expression level of SNHG6 predicted worse progression-free survival in colorectal cancer.

SNHG6 promotes cell growth of colorectal cancer cells in vitro

Next, to determine the functional role of SNHG6 in colorectal cancer, following experiments were performed. Firstly, the expression of SNHG6 in colorectal cancer cell lines was examined. As shown in Fig. 2A, the expression of SNHG6 in colorectal cancer cell lines was relative higher than that in NCM460, a normal human colon mucosal epithelial cell line (*, $p<0.05$). We then chose SW480 and HT-29 cell lines for the following studies. SW480 and HT-29 cell lines with knockdown of the endogenous SNHG6 expression were established by lentiviral transduction (Fig. 2B). The expression of SNHG6 mRNA was significantly repressed by SNHG6 specific shRNAs (*, $p<0.05$). Secondly, the cell proliferation was examined by CCK-8 cell growth assay and colony formation assay. CCK-8 cell growth assay indicated that the proliferation rate of both SW480 and HT-29 cells with knockdown of endogenous SNHG6 was significantly lower than that of control (*, $p<0.05$; Fig. 2C and 2D). The numbers of colonies of vector control, SNHG6-shRNA1 and SNHG6-shRNA2 group in SW480 cells were 107.6 ± 12.4, 76.7 ± 10.1 and 71.7 ± 11.5, respectively (*, $p<0.05$, Fig. 2E). Similar colony formation results were also got from HT-29 cells (*, $p<0.05$, Fig. 2E). Thirdly, we examined the effect of overexpression of SNHG6 on cell proliferation in SW480 and HT-29 cells. Both CCK-8 cell growth assay (Fig. 2F and 2G) and colony formation assay (Fig. 2H) showed that overexpression of SNHG6 enhanced the cell proliferation of SW480 and HT-29 cells (*, $p<0.05$).

Table 1. The relationship between SNHG6 expression and clinical characteristics of patients with colorectal cancer

	SNHG6 expression		
Characteristics	Low(n=33)	High(n=33)	p value
Age (years)			
＜60	7	6	1.000
≥60	26	27	
Gender			
Male	13	11	0.798
Female	20	22	
Tumor location			
Proximal colon	16	14	0.740
Distal colon	10	13	
Rectum	7	6	
Tumor grade			
G1/G2	28	25	0.537
G3/G4	5	8	
Stage			
I / II	26	15	0.011
III / IV	7	18	
Distant metastases			
Yes	2	4	0.672
No	31	29	
Recurrence			
Yes	8	12	0.422
No	25	21	

We further assessed whether SNHG6 could affect DNA synthesis by BrdU incorporation assay in SW480 cells. The results showed that knockdown of SNHG6 dramatically inhibited SW480 cellular DNA synthesis (*, $p<0.05$, Fig. 3A). Furthermore, the effects of SNHG6 on cell cycle progression and cell death were examined in colorectal cancer cells. As shown in Fig. 3B, in the cells with repressed expression of SNHG6, there was a significant increase in the proportion of G1 phase cells and a decrease in S phase cells (*, $p<0.05$). It could thus be inferred that knockdown of endogenous SNHG6 might cause cell cycle arrest in G1 phase in colorectal cells. Cell death was assessed by Annexin V/PI staining assay. Results showed that knockdown of endogenous SNHG6 expression didn't cause cell death in colorectal cancer cells (NS, not significant; Fig. 3C). Lastly, we examined the effect of overexpression of SNHG6 on DNA synthesis by BrdU incorporation assay in SW480 and HT-29 cells. Results showed that overexpression of SNHG6 dramatically enhanced DNA synthesis in colorectal cancer cells (*, $p<0.05$; Fig. 3D). Collectively, these results suggested that SNHG6 promoted colorectal cancer cell proliferation *in vitro*.

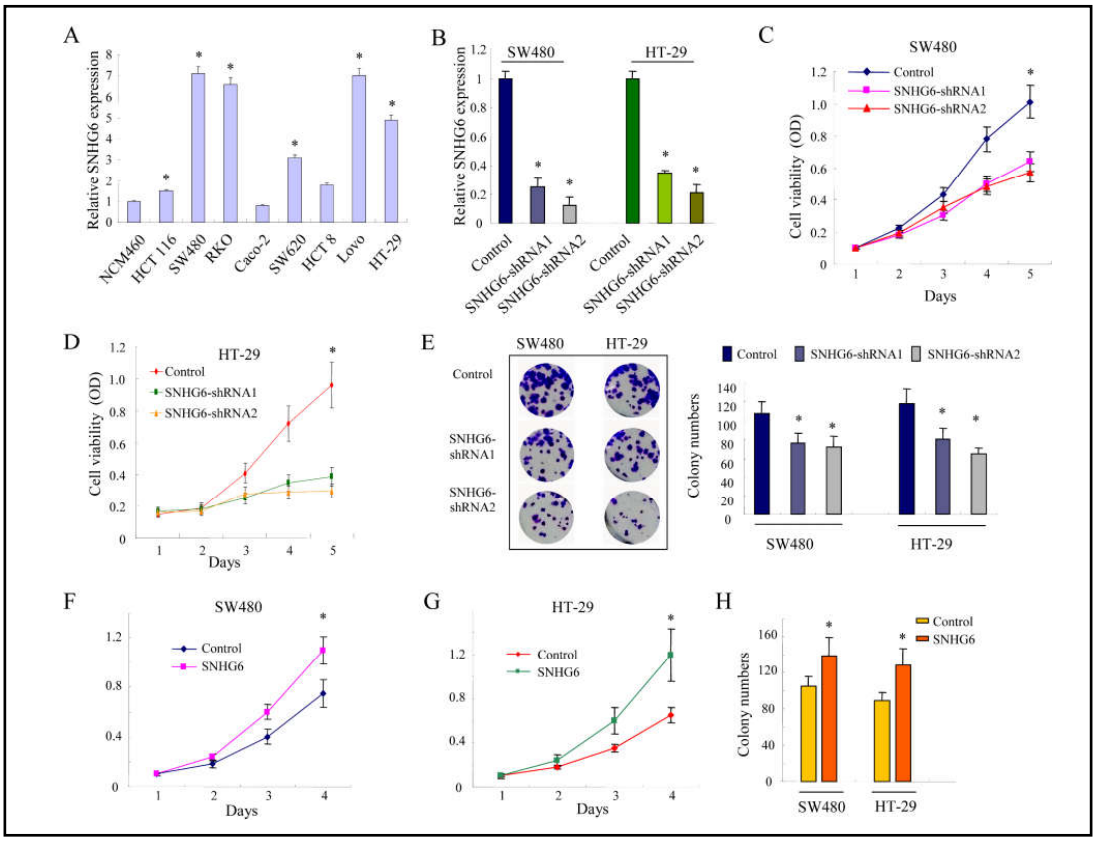

Fig. 2. Knockdown of endogenous SNHG6 suppressed cell growth of colorectal cancer cells in vitro. A. The expression of SNHG6 mRNA in various colorectal cell lines. B. The expression of SNHG6 was examined after knockdown of SNHG6 in SW480 cells. C-E. CCK-8 cell growth assay (C and D) and colony formation assay (E) of both SW480 and HT-29 cells stably transfected either with SNHG6 shRNAs (shRNA-SNHG6-1 and shRNA-SNHG6-2) or scramble control. All experiments were performed in triplicate; bars, s.e.m.; *, p<0.05. F-H. CCK-8 cell growth assay (F and G) and colony formation assay (H) of both SW480 and HT-29 cells stably transfected either with SNHG6 or vector control. All experiments were performed in triplicate; bars, s.e.m.; *, p<0.05.

SNHG6 promotes cell growth of colorectal cancer cells in vivo

We further performed *in vivo* xenograft tumor assay to validate the above findings. SW480 cells with stable expression of SNHG6-shRNAs or vector control were injected subcutaneously into three groups of nude mice. As shown in Fig. 4A, tumors derived from SNHG6-shRNAs group grew significant slowly than those from the vector control group as assessed by tumor volume. The mean tumor volume of vector control, SNHG6-shRNA1 and SNHG6-shRNA2 group were (0.48 ± 0.06) cm^3, (0.20 ± 0.06) cm^3 and (0.24 ± 0.07) cm^3 after four weeks, respectively (*, $p<0.05$). The average tumor weight of vector control, SNHG6-shRNA1 and SNHG6-shRNA2 group were (0.51 ± 0.05) g, (0.21 ± 0.02) g and (0.22 ± 0.03) g, respectively (*, $p<0.05$, Fig. 4B). Ki67 staining assay showed that the Ki67 positive tumor cells was much less in SNHG6-shRNAs groups as compared with the vector control group (*, $p<0.05$, Fig. 4C). In addition, the effect of SNHG6 on cellular apoptosis was explored by cleaved caspase-3 immunochemical staining. However, no significances were found between SNHG6-shRNAs group and control group (NS, not significant; Fig. 4D). Collectively, these results demonstrated that SNHG6 promoted cell growth of colorectal cancer *in vivo*.

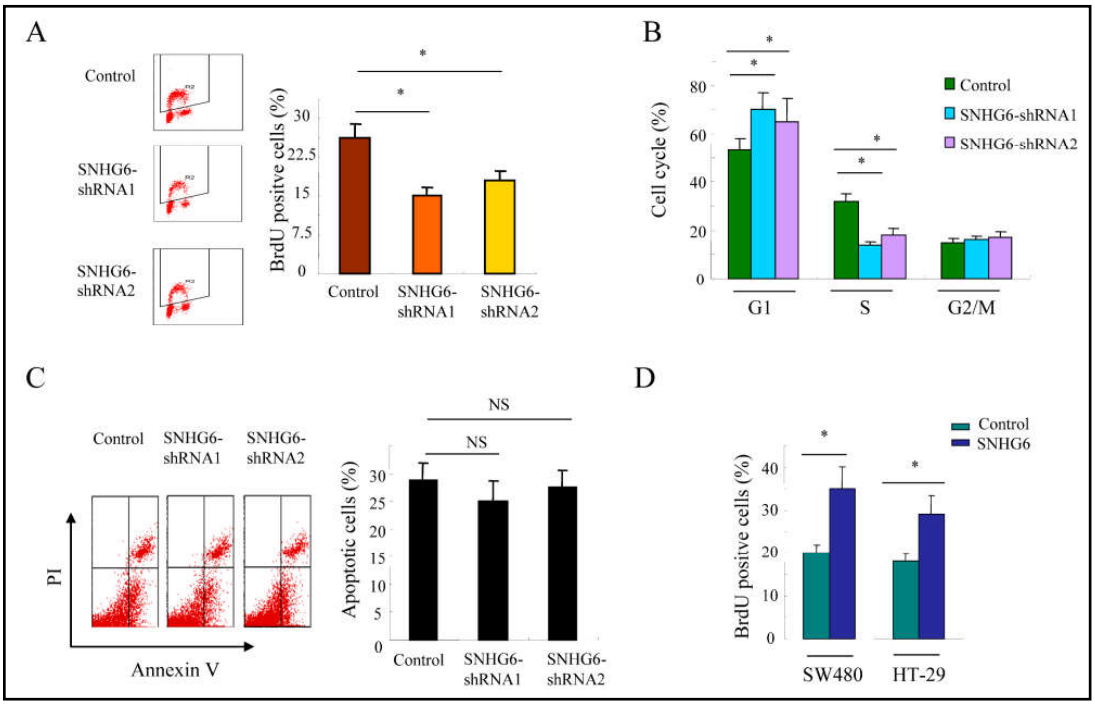

Fig. 3. SNHG6 promoted cell proliferation of colorectal cancer cells in vitroA. BrdU corporation assay of SW480 cells stably transfected either with SNHG6 shRNAs (shRNA-SNHG6-1 and shRNA-SNHG6-2) or scramble control. B. Cell cycle was analyzed after knockdown of SNHG6 in SW480 cells. C. Cell death assay were examined after knockdown of SNHG6 in SW480 cells. Cells were cultured in serum free medium for 36 hours and then subjected for Annexin V/PI staining assay. D. BrdU corporation assay of SW480 cells stably transfected either with SNHG6 or vector control. All experiments were performed in triplicate; bars, s.e.m.; *, p<0.05. NS, not significant.

SNHG6 inhibits p21 expression in colorectal cancer cells

The human cell cycle inhibitor p21 plays a critical role in the tumorigenesis and progression of colorectal cancer [25]. In our study, we found that p21 expression was suppressed by SNHG6 in colorectal cancer cells. First, we examined the changes of p21 mRNA by real-time PCR after knockdown of endogenous SNHG6 in two different colorectal cancer cell lines SW480 and HT-29. The results demonstrated that the expression of p21 was significantly higher in cells transduced with SNHG6-shRNAs than that of cells transduced with scramble control (*, $p<0.05$, Fig. 5A). Second, the protein change of p21 was evaluated by western blot. As shown in Fig. 5B, the expression of p21 was dramatically increased in both SW480 and HT29 cells after knockdown the endogenous SNHG6. We also examined the protein changes of p21 in the xenograft tumor tissues by immunohistochemistry analysis. As compared with scramble control group, p21 expression was significant higher in SNHG6-shRNA1 group (Fig. 5C). Then, the co-expression of SNHG6 and p21 in five colorectal cancer datasets (TCGA, GSE14333, GSE37892, GSE20916 and GSE18105) was analyzed through querying the open database ChIPBase v2.0 and the R2 Genomics Analysis and Visualization Platform (http://r2.amc.nl). The results showed a negative relationship between SNHG6 and p21 in colorectal cancers (TCGA, r: -0.377, p=1.2E-12; GSE14333, r: -0.265, p=4.2E-07; GSE37892, r: -0.358, p=2.9E-05; GSE20916, r: -0.304, p=2.0E-04; and GSE18105, r: -0.763, p=2.2E-22; Fig. 5D).

EZH2 is the catalytic subunit of PRC2, and which plays important roles in catalyzing trimethylation of histone H3 at lysine 27 (H3K27me3) and repressing gene transcription [26, 27]. It has been previously reported that lncRNAs could bind to EZH2 and epigenetically silence the downstream target genes [26, 28]. We therefore performed RNA immunopreciptation (RIP) analysis to examine whether SNHG6 could bind to EZH2. As shown in Fig. 5E, the

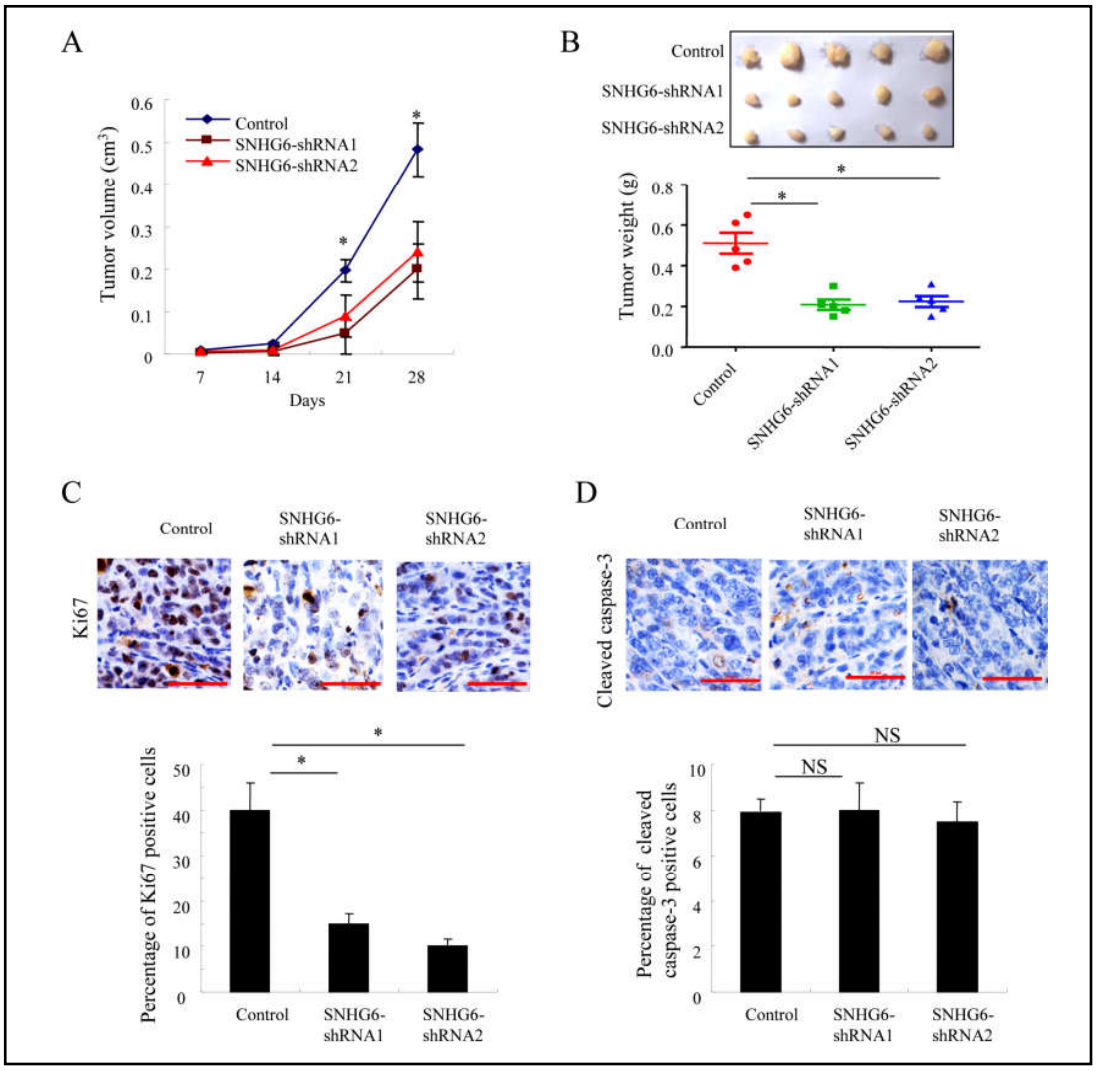

Fig. 4. SNHG6 promotes cell growth of colorectal cancer in vivo. A. in vivo xenograft tumor model. SW480 cells with stable expression of either SNHG6 shRNAs (shRNA-SNHG6-1 and shRNA-SNHG6-2) or scramble control was subcutaneously injected into three groups of nude mice. Tumor volume was measured every week and was calculated using the formula: (Length × Width^2)/2. B. Representative subcutaneous tumor xenografts and the weight of the tumors. C, D. Ki67 (C) and cleaved caspase-3 (D) immunochemical staining assay were conducted in xenograft tumor samples. Scale bars = 50 μm. Bars, s.e.m.; *, p<0.05.

endogenous SNHG6 was enriched in the anti-EZH2 RIP fraction in comparison with IgG control in SW480 cells (*, p<0.05, Fig. 5E). The GAPDH was used as a negative control. Studies have shown that SNHG6 could bind with EZH2 in colorectal cancer cells. Therefore, we examined whether SNHG6 could repress p21 transcription by recruiting EZH2 to the p21 promoter. Chromatin immunoprecipitation (ChIP) assays in SW480 cells were conducted. The results revealed that EZH2 could directly bind to p21 promoter regions and induce H3K27me3 modification in SW480 cells. As compared to control cells, knockdown of SNHG6 significantly decreased the binding of EZH2 and H3K27me3 levels in p21 promoters (*, p<0.05, Fig. 5F). These results suggest that SNHG6 repressed p21 transcription through recruiting EZH2 to the p21 promoter in colorectal cancer cells.

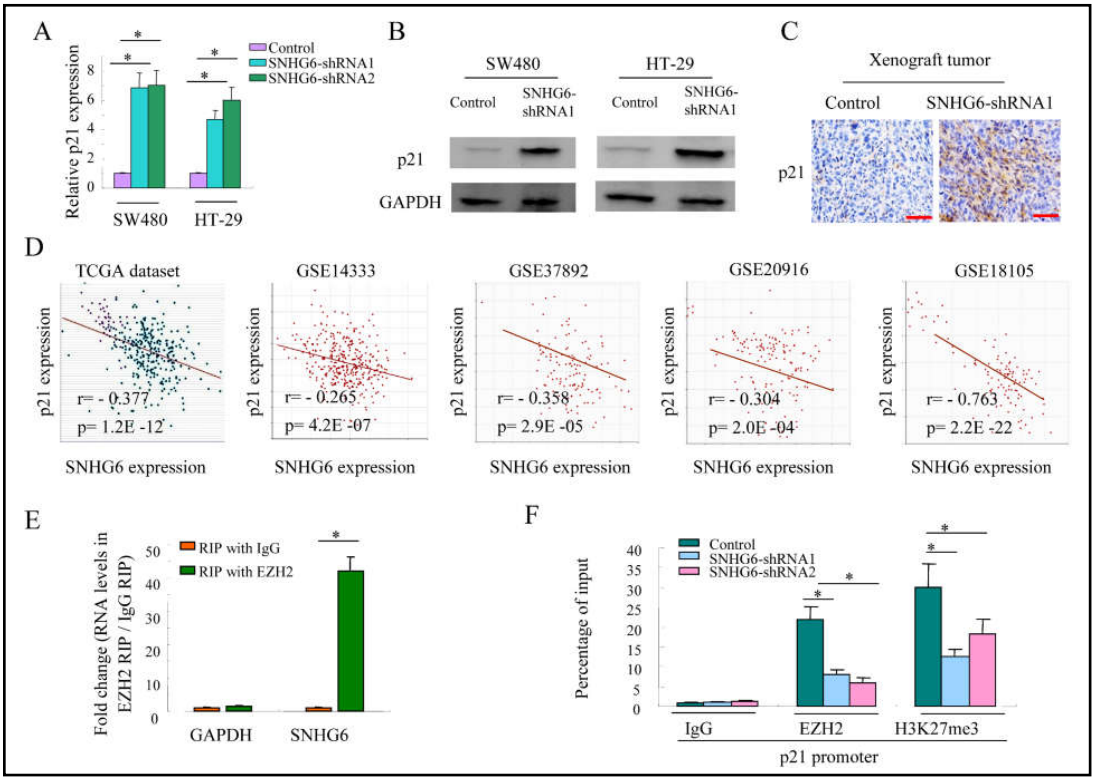

Fig. 5. SNHG6 inhibits p21 expression in colorectal cancer cells. A. Changes of p21 mRNA by real-time PCR after knockdown of endogenous SNHG6 in two different colorectal cancer cell lines SW480 and HT-29. B. Changes in the expression of p21 protein affected by SNHG6 in colorectal cancer cells. C. Changes in the protein levels of p21 in xenograft tumor tissues measured by immunohistochemistry analysis. D. Co-expression analysis of SNHG6 and p21 in TCGA colorectal cancer microarray datasets using the gene correlation module of the R2 microarray analysis and visualization platform. E. RIP analysis was performed to examine whether SNHG6 could bind to EZH2 in SW480 cells. The GAPDH was used as a negative control. F. The binding of EZH2 to the p21 promoter was examined by ChIP analysis. All experiments were performed in triplicate; Scale bars = 50 μm. bars, s.e.m.; *, p<0.05.

SNHG6 promotes cell proliferation via repression of p21 in colorectal cancer

Next, we investigated whether SNHG6 promoted cell proliferation by repression of p21 in colorectal cancer cells. To this end, the endogenous p21 was knocked down in SW480 cells with stable expression of SNHG6-shRNAs. Then cell growth assay, cell cycle analysis and colony formation assay were performed. As a result, cell growth assay demonstrated that knockdown of SNHG6 significantly suppressed cell proliferation; however, this growth suppression effect of SNHG6 knockdown was compromised by repression of p21 in colorectal cancer cells (*, $p<0.05$, Fig. 6A). Cell cycle analysis showed that in cells with repressed expression of SNHG6, there was a significant increase in the proportion of G1 phase cells and a decrease in the proportion of S phase cells, however, this changes could be reversed by inhibition of p21 (*, $p<0.05$, Fig. 6B). The colony numbers of vector control group, SNHG6-shRNA1 group, SNHG6-shRNA1+p21-shRNA1 and SNHG6-shRNA1+p21-shRNA2 group were (105.0±7.2), (72.3±3.5), (94.0±9.2) and (99.7±4.5), respectively (*, $p<0.05$, Fig. 6C). We further performed *in vivo* xenograft tumor assay to validate the above findings. As shown in Fig. 6D, knockdown of SNHG6 suppressed the tumor growth; however, this tumor suppressive effect conferred by SNHG6-shRNA1 was impaired after knockdown of endogenous p21 expression. The mean tumor volume of vector control, SNHG6-shRNA1 and SNHG6-shRNA1+ p21-shRNA1 group were (0.51 ± 0.04) cm^3, (0.25 ± 0.06) cm^3 and (0.45 ± 0.06) cm^3 after four weeks, respectively (*, $p<0.05$, Fig. 6D). The average tumor weight of

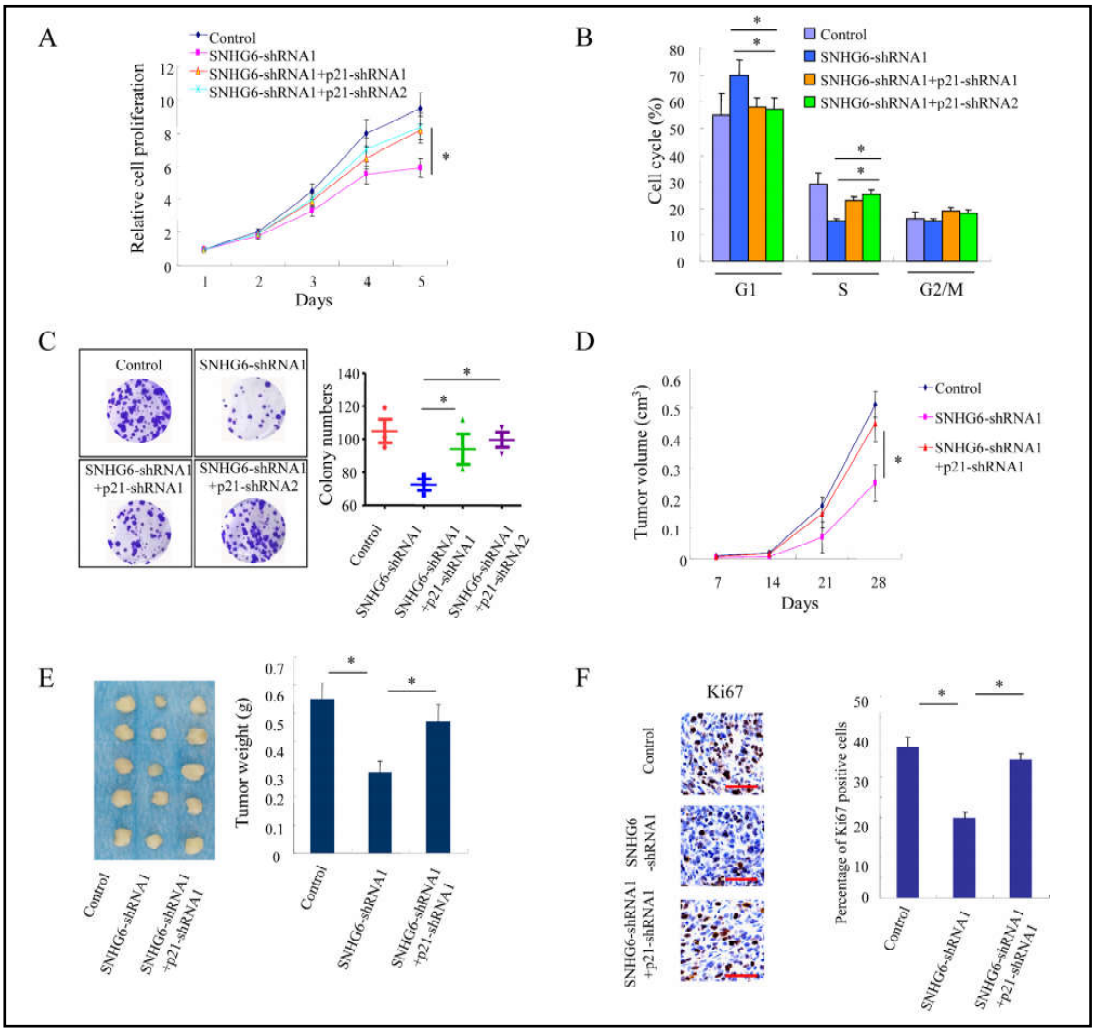

Fig. 6. SNHG6 promotes cell proliferation via repression of p21 in colorectal cancer. The endogenous p21 was knocked down with stable expression of SNHG6-shRNAs in SW480 cells, and then cell growth assay (A), cell cycle analysis (B), colony formation assay (C) xenograft tumor formation assay (D and E), and Ki67 immunochemical staining (F) were performed. All experiments were performed in triplicate; Scale bars = 50 μm. bars, s.e.m.; *, p<0.05.

vector control, SNHG6-shRNA1 and SNHG6-shRNA2 group were (0.55 ± 0.06) g, (0.29 ± 0.04) g and (0.47 ± 0.06) g, respectively (*, p<0.05, Fig. 6E). Similarly, the Ki67 positive tumor cells were reduced after knockdown of SNHG6, and this changes could be reversed by inhibition of p21 (*, p<0.05, Fig. 6F). In summary, these results suggested that SNHG6 promoted cell proliferation via repression of p21 in colorectal cancer.

Discussion

The development and advancement of next-generation sequencing (NGS) technologies to various cancers has revealed thousands of ncRNAs has been revealed whose aberrant expression is associated with different cancer types [29-31]. An increasing number of studies indicate that diverse ncRNAs play a critical role in malignant transformation [32, 33]. SNHG6 is a novel snoRNA host gene [34, 35], but its cellular functions are largely unknown. Aberrantly expression of SNHG6 has been detected in hepatocellular carcinoma and gastric cancer [34, 36, 37]. SNHG6

functions as a competing endogenous RNA to promote cancer progression [34, 36] and regulates ZEB1 expression by competitively binding miR-101-3p and interacting with UPF1 in hepatocellular carcinoma [37]. In addition, SNHG6 is associated with poor prognosis and enhanced tumor growth and epithelial-mesenchymal transition via suppressing p27 and sponging miR-101-3p in gastric cancer [38].

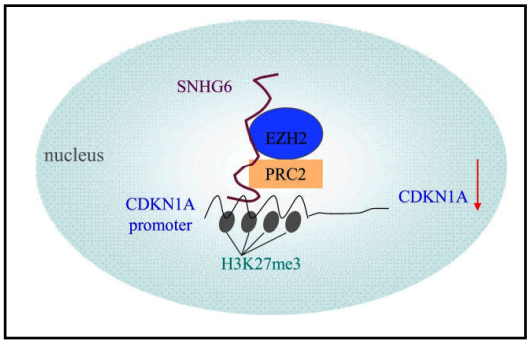

Fig. 7. Schematic figure on illustrate the CDKN1A/p21/SNHG6 interactions.

We here showed that SNHG6 was overexpressed in colorectal cancers and high level of SNHG6 was associated with advanced tumor stage in colorectal cancers, which is in consistent with the above findings. Functional studies demonstrated that SNHG6 promoted colorectal cancer cell growth *in vitro* and *in vivo*. These data suggested that SNHG6 might have an oncogenic role in tumorigenesis of colorectal cancer.

The cyclin-dependent kinase inhibitor p21 could inhibit cell cycle progression by suppression of CDK2 activity [19, 39, 40], and its role in the tumor development and progression has been widely studied [41, 42]. It has been suggested that p21 may act as a tumor suppressor in colorectal cancer [43, 44]. Loss of expression or function of p21 has been implicated in the genesis or progression of a variety of human cancers [13, 41, 45]. In the present study, we showed that p21 expression could be inhibited by SNHG6 in colorectal cancer cells, and the repression of p21 was essential for SNHG6 to promote tumor cell growth in colorectal cancer. Hence, we come to the conclusion that SNHG6 promotes colorectal cancer cell growth by repressing transcription of p21, which is in line with previous studies.

With the progression of studies about lncRNAs, more and more ncRNAs have been found to play a critical role in tumor development and progression, however, the underlying molecular mechanisms underlying the effects of ncRNA are largely unknown [7]. These RNAs may work in gene regulatory networks as signals, decoys, guides or scaffold [46-48], and coordinate histone modifications by binding to various histone modification enzymes such as PRC2, which is a methyltransferase for H3K27me3 and is involved in the repression of gene transcription [49-51]. EZH2 is a catalytic subunit of PRC2[52]. Some ncRNAs could bind with EZH2 to epigenetically inhibit downstream target genes [49]. Our data showed that SNHG6 repressed p21 transcription through recruiting EZH2 to the p21 promoter in colorectal cancer cells (Fig. 7), implicating the important role of EZH2 in colorectal cancer development.

Conclusion

Our study demonstrates that aberrantly expression of SNHG6 promotes cell growth through downregulation of p21 in colorectal cancer cells. Moreover, SNHG6 repressed p21 transcription by recruiting EZH2 to the p21 promoter. Survival analysis reveals that colorectal cancer patients with high SNHG6 expression have a poor progress-free survival. Taken together, our study not only yields a better understanding of the role of SNHG6 in colorectal tumorigenesis and cancer aggression, but also provides a promising target for novel anticancer therapeutics.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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