European Review for Medical and Pharmacological Sciences 2019; 23: 5705-5713

Long non-coding RNA GClnc1 promotes progression of colorectal cancer by inhibiting p53 signaling pathway

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Abstract. – OBJECTIVE: The aim of this study was to investigate whether long non-coding RNA (lncRNA) GClnc1 was involved in the development of colorectal cancer, and to explore its possible mechanisms.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was conducted to detect GClnc1 expression in 48 colorectal cancer tissues and normal colon tissues. The Kaplan-Meier method was used to analyze the relationship between GClnc1 expression and survival rate of patients with colorectal cancer. In addition, GClnc1 expression in colorectal cancer cell lines and normal colonic epithelial cell lines were analyzed. After knockdown and over-expression of GClnc1 in colorectal cancer cells, Cell Counting Kit-8 (CCK-8) and colony formation assay were performed to detect the viability and proliferation of cells, respectively. RNA pull-down and RNA-binding protein immunoprecipitation (RIP) were applied to examine the specific interaction between GClnc1 and p53. After over-expression of GClnc1 in colorectal cancer cells, qPCR and Western blot were performed to evaluate the expression levels of p53, p21 and BAX. Meanwhile, the Luciferase reporter gene assay was established to reveal the activity of p53 after over-expression of GClnc1. ChIP assay was applied to figure out whether GClnc1 could affect the binding ability of p53 to the promoter region of p21. After p53 or GClnc1 knock-down in colorectal cancer cells, the protein level of p53 was analyzed using Western blot. Finally, qRT-PCR, CCK-8 and colony formation assay were used to detect the levels of p21 and BAX, the viability, as well as the proliferation ability of cells, respectively.

RESULTS: The expression of GClnc1 in colorectal cancer tissues was significantly higher than that of para-cancerous tissues. Meanwhile, GClnc1 expression in T3 and T4 tumors was markedly higher than that of T1 and T2. The survival analysis revealed that patients with a higher level of GClnc1 showed remarkably lower overall survival than those with lower expression of GClnc1. QRT-PCR results indicated that GClnc1 expression in colorectal cancer cells (including SW620 and HCT116) was conspicuously higher than that of normal colonic epithelial cells (NCM640). After knocking down GClnc1 in SW620 cells, the viability and proliferation abilities were conspicuously decreased. Meanwhile, the expression level of GClnc1, as well as the viability and colony formation ability of cells, were significantly increased after over-expression of GClnc1 in HCT116 cells. Subsequently, the qRT-PCR assay demonstrated that GClnc1 was mainly localized in the nucleus. RNA pull-down and RIP experiments revealed that there was a specific interaction between GClnc1 and p53. Moreover, qRT-PCR and Western blot analysis indicated that the expression level of p53 was not affected after over-expression of GClnc. However, the expressions of p21 and BAX were remarkably decreased. The Luciferase reporter gene assay revealed that GClnc1 over-expression markedly weakened the Luciferase activity of p53. Meanwhile, ChIP experiments demonstrated that GClnc1 up-regulation affected the binding condition of p53 to p21. Western blot analysis showed that knockdown of p53 reversed the increased mRNA level of p21 as well as BAX. Furthermore, p53 down-regulation significantly weakened cell viability and colony formation ability caused by knockdown of GClnc1.

CONCLUSIONS: LncRNA GClnc1 was highly expressed in colorectal cancer tissues. Meanwhile, it could increase the proliferation of colorectal cancer cells by reducing the expression of p21 as well as BAX via p53 signaling pathway, thereby promoting the progression of colorectal cancer.

Key Words:
Colorectal cancer, GClnc1, p53, Proliferation.

Introduction

Colorectal cancer (CRC) is one of the most common cancers in the world, which is also the

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third leading cause of cancer-related deaths[1]. According to the 2017 colorectal cancer statistics, there were approximately 135,430 newly diagnosed CRC cases and 50,260 deaths in the United States[2]. In recent years, the incidence and mortality of CRC in China have been greatly increased[3]. There are a variety of risk factors for CRC, such as older age, obesity, lack of physical exercise, smoking, etc. Jeon et al[4] have demonstrated that genetic factors play an important role in CRC carcinogenesis. With in-depth research on the pathogenesis of CRC, targeted therapy has recently played an increasingly important role in the treatment of CRC. However, its therapeutic effect is far from satisfactory[5].

Long non-coding RNAs (lncRNAs) are a kind of RNAs with about 200 nt to 100 000 nt in length. They can regulate the gene expression at the epigenetic level[6,7]. Some studies[8,9] have revealed that lncRNA plays a vital role in various cancers, including bladder cancer, prostate cancer, kidney cancer, breast cancer, lung cancer, etc. Currently, it has been found that lncRNA GClnc1 plays a crucial role in the development of gastric cancer[10]. In addition, some researchers have also revealed the potential role of lncRNA in the diagnosis, prognosis and treatment of CRC. However, the specific role of lncRNA GClnc1 in CRC has not been fully elucidated[11].

P53 is about 16-20 kb in length, which is a pair of alleles. P53 acts as a tumor suppressor gene localized on human chromosome 17[12]. It can inhibit cell growth and induce cell apoptosis in cellular stress responses, eventually playing a key part in preventing canceration of damaged cells[13]. An impaired p53 function has been found in multiple human cancers[14]. Furthermore, other studies have indicated that p53 mutation in breast cancer is associated with low survival rate and malignancy of the tumor[13].

In this work, we investigated whether long non-coding RNA GClnc1 could promote the progression of CRC by inhibiting the p53 signaling pathway.

Patients and Methods

Clinical Samples Collection

Tumor tissues and adjacent normal tissues were obtained from 48 CRC patients who underwent surgical resection from February 2008 to October 2015 in our hospital. All surgically removed tissue samples were rapidly frozen in liquid nitrogen for subsequent use. Inclusion criteria for CRC patients included no history of treatment, no history of other malignant tumors, and CRC confirmed by histopathology. This study was approved by the Second Affiliated Hospital of Zhengzhou University Ethics Committee. Written informed consent was obtained from each patient or their guardian before the study.

Cell Culture

Colorectal cancer cells (SW620 and HCT116) and normal colonic epithelial cells (NCM640) were obtained from the Shanghai Cell Bank (Shanghai, China). All cells were cultured in Roswell Park Memorial Institute-1640 medium (RPMI-1640; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 1% streptomycin and penicillin, and maintained in a saturated humidity incubator with 5% CO_2 at 37°C. Subsequently, the cells were digested, seeded into appropriate cell dishes, and cultured in an incubator until 60% of cell density.

Cell Transfection

Cells were first inoculated into 6-well plates. When the cell density reached 60%, the cells were transfected with si-GClnc1, si-p53, pcDNA-GClnc1, as well as corresponding negative controls according to the instructions of Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA). The culture medium was replaced 6 h later, and the cells were collected for subsequent experiments.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) Detection

Total RNA in BMSCs was extracted by TRIzol method (Invitrogen, Carlsbad, CA, USA). Then, the concentration of extracted RNA was measured using an ultraviolet spectrophotometer (Hitachi, Tokyo, Japan). Subsequently, RNA was reverse transcribed into complementary deoxyribose nucleic acid (cDNA), and the SYBR Green method was used for PCR detection. Three replicates were set in each group. Specific quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) reaction parameters were as follows: denaturalization at 95°C for 60 s, extension at 95°C for 30 s and annealing at 60°C for 40 s, for a total of 40 cycles. The expressions of lnc-GClnc1, p53, p21, BAX were examined. The primer sequences used in this study were shown in Table I.

Table I. Primer sequences.

U6	F:5'-CTCGCTTCGGCAGCACA-3'
	R:5'-AACGCTTCACGAATTTGCGT-3'
p21	F:5'-GGCAGACCAGCATGACAGATT-3'
	R:5'-GCGGATTAGGGCTTCCTCT-3'
p53	F:5'-CCCAAGCAATGGATGATTTGA-3'
	R:5'-ATGAGGGTGCTGTCTTTGTAGG-3'
BAX	F:5'-CATGTTTTCTGACGGCAACTT-3'
	R:5'-CCAGATCACGCCATTTCAC-3'
GClnc1	F:5'-TGGGGTAACTTAGCAGTTTCAAT-3'
	R:5'-GGCAAGCAGTAATCTTACATGACAC-3'
GAPDH	F:5'-CGGAGTCAACGGATTTGGTCGT-3'
	R:5'--GGGAAGGATCTGTCTCTGACC-3'
β-actin	F:5'-AGAGCCTCGCCTTTGCCGAT-3'
	R:5'-CCATCACGCCCTGGTGCCT-3'

Cell Counting Kit-8 (CCK-8) Assay

Transfected cells were first seeded into 96-well plates for 24 h of culture. Subsequently, 10 uL of Cell Counting Kit-8 reagent (CCK-8; Dojindo, Kumamoto, Japan) was added in each well, followed by incubation for 1 h at 37°C. Finally, the optical density (OD) value at 450 nm was measured by a microplate reader.

Colony Formation Assay

Cells in the logarithmic growth phase were first digested with trypsin and then made into a single cell suspension. A total of 2500 cells were plated into 6-well culture plates, with three replicate wells in each group. After culture for 7 days, the cells were fixed with 95% ethanol and stained with 1% crystal violet. Finally, the cells were photographed under a microscope, and the number of formed colonies was counted.

Subcellular Localization Analysis

RNA was extracted from the nucleus or cytoplasm of HCT116 cells according to the instructions of the PARIS kit (Invitrogen, Carlsbad, CA, USA). Total and nuclear RNA were then extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Finally, the mRNA expressions of GClnc1, U6 RNA (nucleus), β-actin and GAPDH (cytoplasm) were detected by qRT-PCR analysis.

Luciferase Reporter Gene Assay

Cells at a density of 3 x 10^5 cells per well were first seeded into 24-well plates. Then, the cells were co-transfected with wild-type or mutant psiCHECK-2 p53 vector (Generay, Shanghai, China) and pcDNA-GClnc1, pcDNA-NC in strict accordance with Lipofectamine 2000. 24 h after transfection, the Luciferase activity of the cells was measured using a Dual-Luciferase reporter assay kit.

Western Blot

Total protein extracted from cells was separated by electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking for 2 h at room temperature, the membranes were incubated with primary antibodies overnight. On the next day, the membranes were incubated with the corresponding secondary antibody at room temperature for 2 hours. Finally, immunoreactive protein bands were detected by chemiluminescence method (ECL; Thermo Fisher Scientific, Waltham, MA, USA).

RNA Pull-Down Experiment

To determine the interaction between GClnc1 and p53, pGEM-T vector (Promega, Madison, WI, USA) carrying GClnc1, the anti-sense of GClnc1 DNA was linearized with corresponding restriction enzymes. Subsequently, template DNA was prepared for *in vitro* transcription. Biotinylated RNA transcript was then transcribed using T7 RNA polymerase (Roche, Basel, Switzerland). 3 mg of biotinylated RNA was mixed with proteins extracted from cells. After that, RNA was targeted with streptavidin beads (Millipore, Billerica, MA, USA). Finally, the co-precipitated protein was visualized by Western blotting.

RNA-Binding Protein Immunoprecipitation (RIP) Test

RNA immunoprecipitation (RIP) experiment was performed according to the instructions of the Magna RIP RNA Binding Protein Immunoprecipitation Kit (Sigma-Aldrich, St. Louis, MO, USA). After obtaining cell lysates, magnetic beads were prepared and re-suspended in Wash Buffer, followed by incubation on ice. RNA binding protein immunoprecipitation was then performed. RNA purification was carried out by phenol, chloroform, Salt Solution I, Salt Solution II, Precipitate Enhancer, absolute ethanol (no RNase), dissolved in 10-20 uL of diethyl pyrocarbonate (DEPC) water (Beyotime, Shanghai, China), and stored at -80°C. Finally, the expression of GClnc1 in co-p53 protein and IgG protein precipitate was detected by qRT-PCR.

Chromatin Immunoprecipitation (CHIP)

P53 chromatin immuno-precipitation was processed using Magna ChIP A/G One-Color

Chromatin Immunoprecipitation Kit (Millipore, Billerica, MA, USA). Chromatin immunoprecipitated DNA was eluted, reverse X-linked, purified and analyzed by qRT-PCR.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 16.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. Experimental data of each group were expressed as mean ± standard deviation ($\bar{x}\pm s$). *t*-test was applied to compare the difference of quantitative data between the two groups. Cumulative survival rates were assessed using the Kaplan-Meier method, and the differences were determined by the log-rank test. $p<0.05$ was considered statistically significant: $^{*}p<0.05$.

Results

GClnc1 Is Highly Expressed in Colorectal Cancer and Is Associated With Poor Prognosis

To clarify the relationship between the expression of GClnc1 and the development of CRC, we first detected the expression level of GClnc1 in CRC tissues and normal colon tissues by qRT-PCR. The results showed that the level of GClnc1 in CRC tissues was conspicuously higher than that of normal colon tissue (Figure 1A). After paired analysis of tissue samples, we found that the majority of patients with CRC had high expression of GClnc1 (Figure 1B). At the same time, we found that the expression of GClnc1 in T3 and T4 tumors was markedly higher than that of T1 and T2 (Figure 1C). The Kaplan-Meier analysis showed that CRC patients with higher GClnc1expression had lower overall survival than those with lower expression of GClnc1 (Figure 1D). These results indicated that GClnc1 was involved in the progression of CRC and might be associated with poor prognosis.

GClnc1 Promotes the Growth of CRC Cells

To further investigate the role of GClnc1 *in vitro*, we detected the expression of GClnc1 in CRC cells SW620 and HCT116 as well. QRT-PCR results found that GClnc1 expression in CRC cells was significantly higher than that of normal colonic epithelial cells NCM640 (Figure 2A). Subsequently, we knocked down GClnc1 in SW620 cells (Figure 2B). CCK8 and colony formation assay showed that the viability and proliferation abilities of SW620 cells were markedly decreased after knockdown of GClnc1 expression. Next, we over-expressed GClnc1 in HCT116 cells (Figure 2C). Similarly, CCK-8 and colony formation assay demonstrated that the viability and proliferation of HCT116 cells were remarkably increased after over-expression of GClnc1. The above results indicated that high expression of GClnc1 promotes the growth of CRC cells.

GClnc1 Binds to p53 and Inhibits p53 Activity

To further explore how GClnc1 affected the proliferation of colon cancer cells, we performed cell localization of GClnc1. The results found that GClnc1 was mainly expressed in the cytoplasm (Figure 3A). Subsequently, RNA pull-down assay and Western blot were performed to determine the specific interaction between GClnc1 and p53 (Figure 3B, 3C). RIP experiment found that the level of GClnc1 in the p53 antibody precipitation complex was significantly higher than that of the IgG control group (Figure 3D). Next, to investigate whether GClnc1 could inhibit the expression of p53, we over-expressed GClnc1 in HCT116 cells. The results indicated the mRNA and protein expressions of p53 were not significantly changed. However, the expression of p21, as well as BAX, was remarkably reduced (Figure 3E, 3F). Additionally, the Luciferase reporter gene assay revealed that over-expression of GClnc1 could inhibit the Luciferase activity of p53 (Figure 3G). Moreover, ChIP and qRT-PCR assay confirmed that GClnc1 over-expression markedly inhibited the binding condition of p53 to p21. The above results indicated that GClnc1 could affect the levels of p21 and BAX by inhibiting the activity of p53.

GClnc1 Promotes the Growth of CRC Via p53

To confirm that GClnc1 promoted the proliferation of CRC cells by affecting p53 activity, we silenced GClnc1 in HCT116 cells. Western blot results found that the protein level of p53 was not affected. At the same time, we simultaneously knocked down p53 in cells as well (Figure 4A). QRT-PCR results indicated that the expressions of BAX and p21 were significantly increased after knockdown of GClnc1 in HCT116 cells. However, they markedly decreased after knocking down GClnc1 and p53 (Figure 4B). Furthermore, CCK-8 experiments showed that knockdown of p53 remarkably reversed the decrease in cell viability caused by knockdown of GClnc1 (Figure 4C). Col-

ony formation assay demonstrated that knockdown of p53 reversed decreased proliferation of CRC cells resulted from GClnc1 knockdown (Figure 4D). The above results demonstrated that GClnc1 could inhibit the expressions of BAX and p21 by p53 and promote the proliferation of CRC cells.

Discussion

At present, CRC remains a major public health problem in the world[15,16]. In this study, we investigated the relationship between the survival of CRC patients and GClnc1 expression in China. The results indicated that the overall survival of patients with higher GClnc1 expression was conspicuously lower than that of patients with lower expression.

LncRNA has been shown to be involved in chromatin remodeling complexes[17]. However, the underlying molecular mechanisms remain unknown[18]. Zhang et al[9] have indicated that LncRNA is associated with the overall survival of multiple cancer patients and may also act as a biomarker for prognosis of patients with CRC. It has been reported that GClnc1 can change the histone modification pattern of gastric cancer cells. Meanwhile, GClnc1 activates target genes to affect the progression of gastric cancer[11]. We showed that GClnc1 was highly expressed in CRC tissues and

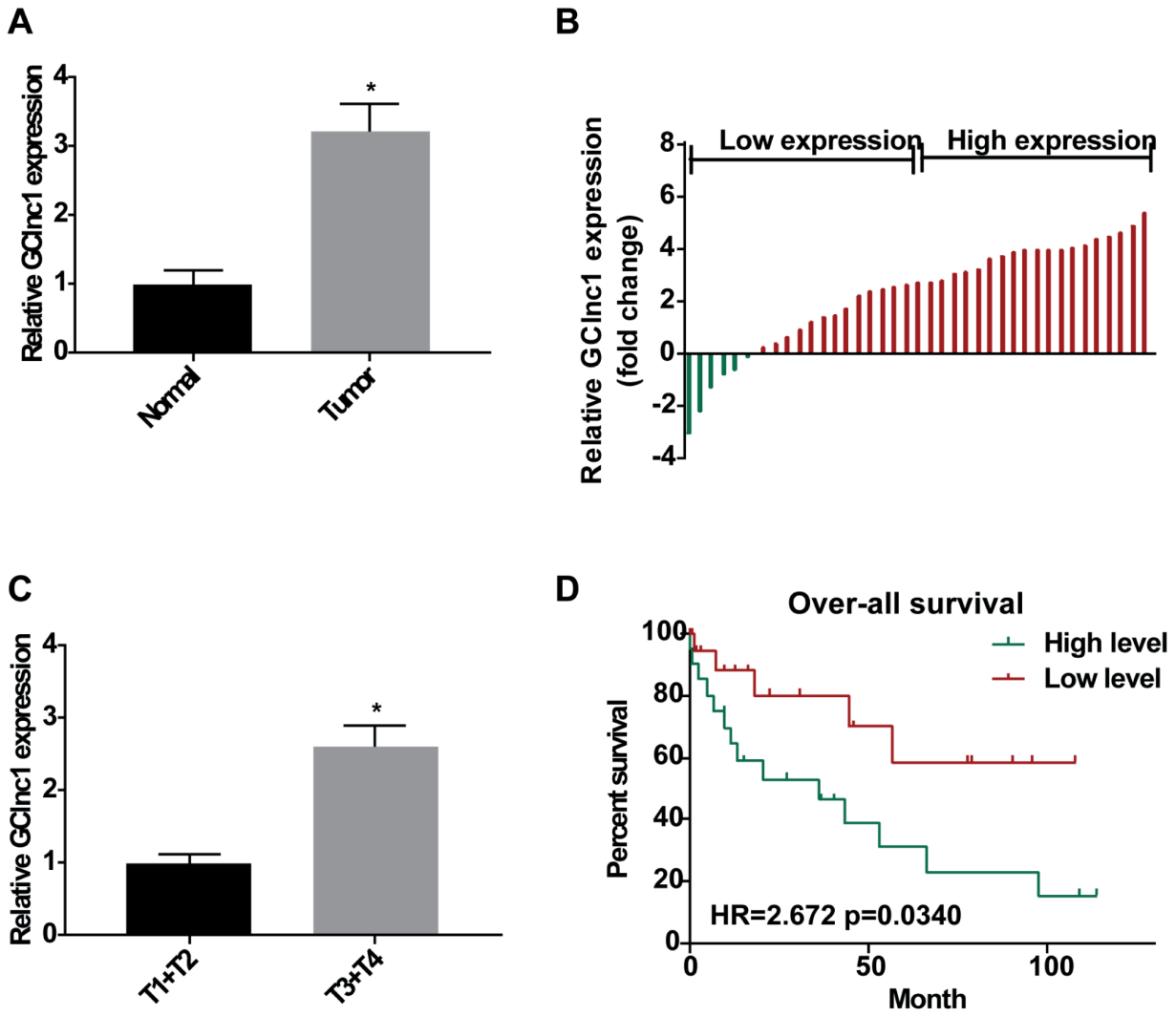

Figure 1. GClnc1 is highly expressed in CRC tissues and is associated with poor prognosis. ***A, B,*** The expression of GClnc1 in 48 CRC patients was significantly higher than that of normal controls. ***C,*** QRT-PCR showed that GClnc1 expression in T3 and T4 tumors was significantly higher than of T1 and T2. ***D,*** The overall survival of patients with higher expression of GClnc1 was markedly lower than that of patients with lower expression.

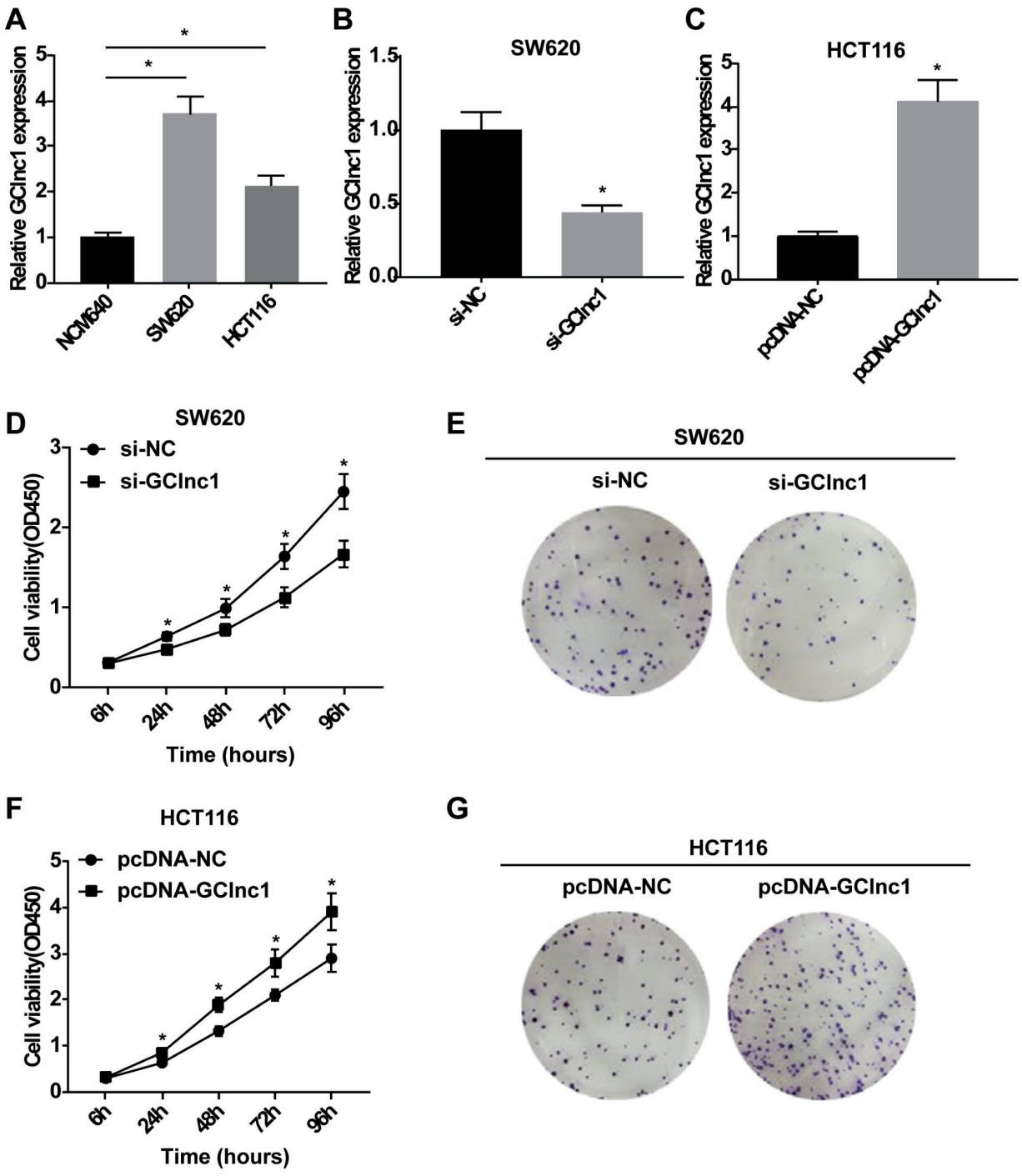

Figure 2. High expression of GClnc1 promotes the proliferation of CRC cells. ***A,*** GClnc1 expression in CRC cells (SW620 and HCT116) was remarkably higher than normal colonic epithelial cells NCM640. ***B,*** After knockdown of GClnc in SW620 cells, the expression of GClnc1 was significantly decreased by qRT-PCR. ***C,*** After over-expression of GClnc1 in HCT116 cells, the expression of GClnc1 was markedly increased by qRT-PCR. ***D,*** After knocking down GClnc1 in SW620 cells, the viability of cells were significantly reduced. ***E,*** Colony formation assay showed that the colony formation ability of cells was remarkably reduced (Magnification × 20). ***F,*** After over-expressing GClnc1 in HCT116 cells, the viability of cells were markedly increased; ***G,*** Colony formation assay showed that the colony formation ability of cells was significantly enhanced (Magnification × 20).

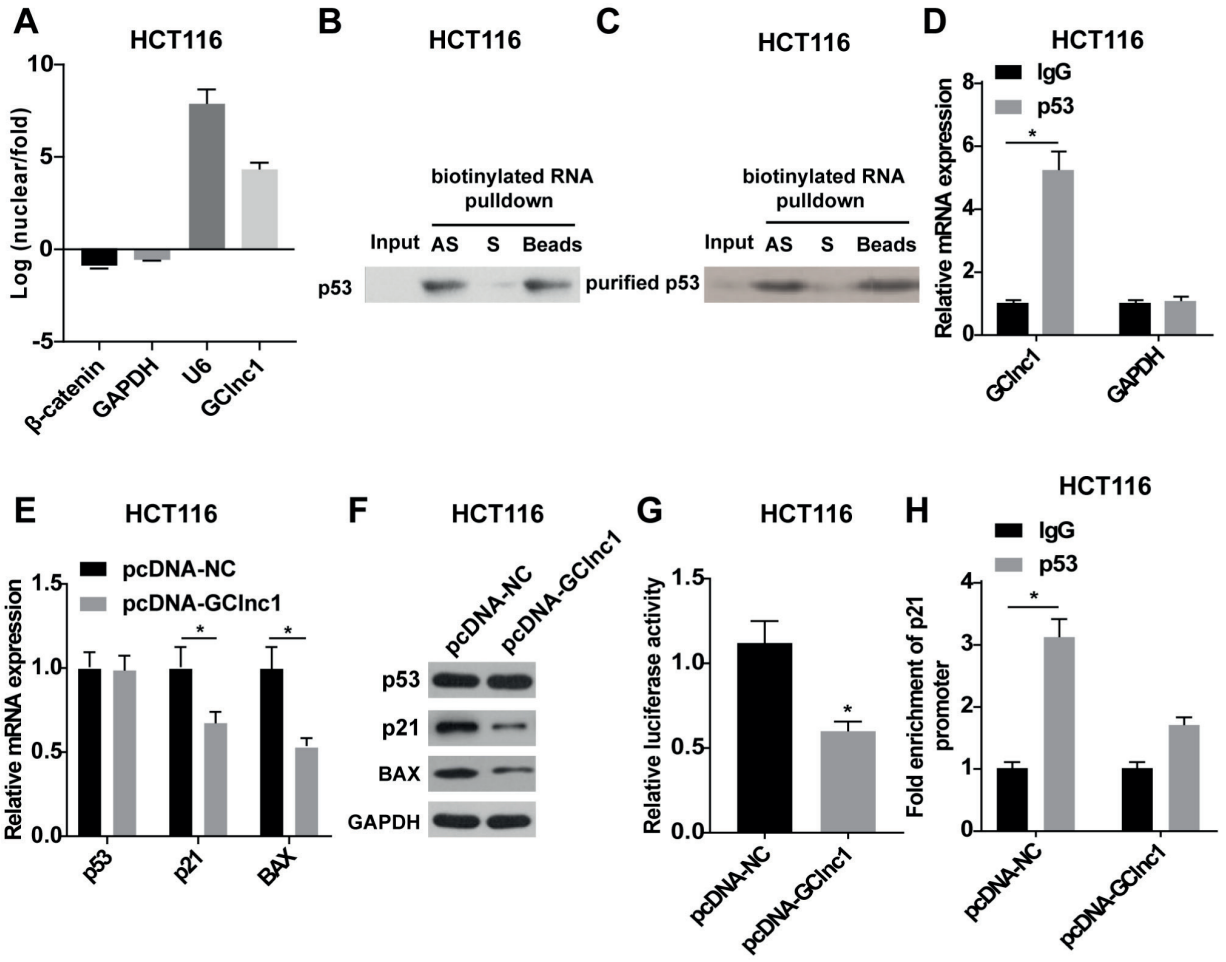

Figure 3. GClnc1 can bind to p53. ***A,*** In HCT116 cells, qRT-PCR detected the expressions of GAPDH, β-catenin, U6 and GClnc1 in the nucleus and cytoplasm. ***B,*** Western blot was used to detect the specific interaction between GClnc1 and p53. ***C,*** Western blot analysis of the specific interaction of GClnc1 with purified p53. ***D,*** RIP and qRT-PCR were used to detect the correlation of p53 with GClnc1 *in vivo*. ***E,*** The mRNA expression levels of p53, p21 and BAX after over-expression of GClnc1 were detected by qRT-PCR. ***F,*** Western blot analysis of the protein expression levels of p53, p21 and BAX after expression of GClnc1. ***G,*** Luciferase reporter gene assay demonstrated that after over-expression of GClnc1, the activity of p53 was significantly decreased. ***H,*** ChIP and qRT-PCR experiments showed that GClnc1 overe-xpression affected the binding ability of p53 to the promoter region of p21.

was associated with poor prognosis of CRC patients. Besides, GClnc1 inhibited the expressions of p21 and BAX in CRC cells by affecting p53 activity. All the results suggested that GClnc1 affected the proliferation of CRC cells through p53.

Tumor suppressor p53 affects cell apoptosis and cell cycle arrest, among which BAX and p21 are important mediators[20]. De et al[21] have found that p53 can enter the nucleus to inactivate p21. Meanwhile, by binding to the cyclin-cdk complex, the cell cycle is arrested in the G2/M phase. At the same time, it has been reported[22] that p53 affects the proliferation activity of cancer cells through the BAX/BCL2 pathway. Long non-coding RNAHOXB-AS3 has been observed to promote the proliferation of hepatocellular carcinoma cells and inhibit cell apoptosis by regulating p53 expression[23]. Our experimental results indicated that GClnc1 could inhibit p53 activity in CRC cells. Moreover, knocking down p53 significantly inhibited the expressions of p21 and BAX, as well as enhanced the proliferation ability of CRC cells.

Conclusions

We found that long non-coding RNA GClnc1 could increase the expressions of p21 and BAX

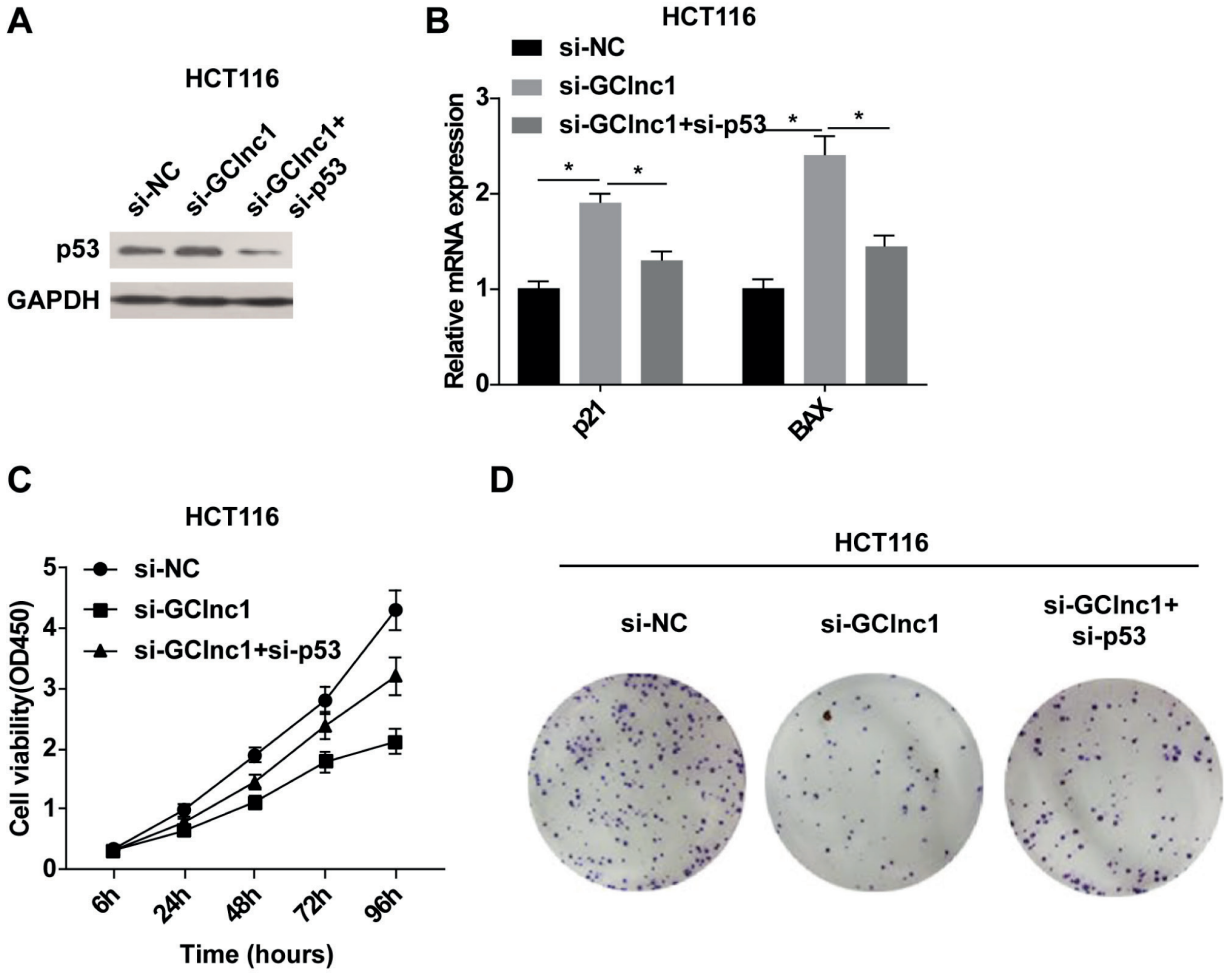

Figure 4. GClnc1 promotes proliferation of CRC cells *via* p53. ***A,*** In HCT116 cells, Western blot showed that the protein expression of p53 was significantly reduced after knockdown of p53. ***B,*** QRT-PCR detected that knockdown of p53 increased the mRNA expressions of p21 and BAX induced by knockdown of GClnc1. ***C,*** CCK-8 showed that knockdown of p53 increased the decrease in cell viability caused by knockdown of GClnc1. ***D,*** Colony formation assay detected that knockdown of p53 reduced the decrease in cell proliferation ability caused by knockdown of GClnc1 (Magnification × 20).

through the p53 signaling pathway. Meanwhile, it significantly increased the proliferation of CRC cells, thereby promoting the progression of CRC. Our findings suggested that GClnc1 might serve as a potential prognostic marker and a new therapeutic target for CRC patients.

Conflict of interest

The authors declare no conflicts of interest.

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