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ORIGINAL ARTICLE

High expression of Plnc RNA-1 promotes the progression of colorectal cancer

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Summary

Purpose: *To study the expression of Plnc RNA-1 in colorectal cancer tissues and cells, and to explore the role of Plnc RNA-1 in the regulation of cell cycle and in the progression of colorectal cancer.*

Methods: *A total of 77 cases of colorectal cancer tissues were retrospectively analyzed. Thirty colorectal normal tissues composed the control group. The expression of Plnc RNA-1 in colorectal cancer tissues at different stages was detected by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) so as to analyze the influence of Plnc RNA-1 on the survival of colorectal cancer patients and the accuracy of colorectal cancer diagnosis. Besides, SW116 and LOVO cell lines were transfected with si-Plnc RNA-1, pc-DNA-Plnc RNA-1 as well as their negative controls to achieve Plnc RNA-1 knockdown or overexpression. Then, we detected the transfection efficiency by qRT-PCR. Furthermore, cell counting kit-8 (CCK-8) and colony formation assays were performed to explore the effect of Plnc RNA-1 on colorectal cancer cells proliferation. Flow cytometry was used to examine the effect of Plnc RNA-1 on the cell cycle. In addition, western blotting was used to detect the expression levels of p-GSK3β, p-Rb and CyclinD1.*

Results: *The expression level of Plnc RNA-1 in 77 colorectal cancer tissues was significantly higher than that of the control. Plnc RNA-1 expression level in patients with infiltrating T3+T4 stages was higher than that in infiltrating T1+T2 stages. In stages III+IV patients, the expression level of Plnc RNA-1 was higher than that of stages I+II. Plnc RNA-1 high expression group exhibited significantly lower survival rate than Plnc RNA-1 low expression group, suggesting there was a significant positive correlation between the sensitivity of colorectal cancer diagnosis and the expression of Plnc RNA-1. Overexpression of Plnc RNA-1 could significantly increase the viability and proliferation in the SW116 cells. In the LOVO cell line, knockdown of Plnc RNA-1 significantly decreased cell viability and proliferation. In the LOVO cell line, knockdown of Plnc RNA-1 promoted cell cycle and decreased the expression of cell cycle-related proteins p-GSK3β, p-Rb and cyclinD1.In the SW116 cell line, overexpression of Plnc RNA-1 led to cell cycle arrest and increased the expression of cell cycle-related proteins.*

Conclusions: *The expression of Plnc RNA-1 in colorectal cancer cells was significantly upregulated. Plnc RNA-1 participated in the development of colorectal cancer through regulating the cell cycle, which may provide a new theoretical basis for the treatment of colorectal cancer and a new therapeutic target.*

Key words: *cell cycle, cell viability, colorectal cancer, Plnc RNA-1*

Introduction

Colorectal cancer is a malignant tumor that seriously threatens the life of people in our country [1]. According to data from the WHO, the mortality rate of colorectal cancer in China increased by 70.7% in 2005 compared with that of 1991, with an average annual increase of 4.71% [2]. The incidence and mortality of colorectal cancer display an increasing tendency, and metastasis is the main

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cause of death [3]. Therefore, studying the specific molecular mechanisms of the development of colorectal cancer could help identify high-risk populations and explore specific molecular markers for early diagnosis, which might help establish new therapeutic targets and exert important significance for improving the efficacy of colorectal cancer therapy [4].

Long non-coding RNA (lncRNA) is a non-coding RNA with a length more than 200 nucleotides (nt) which is usually transcribed in eukaryotes. It can regulate different levels of gene expression as RNA, including pre-transcription, transcription, and post-transcription, but not protein coding. lncRNAs were originally thought to be a byproduct of RNA polymerase II transcription without any biological function [5]. In recent years, studies have shown that many lncRNAs can maximally change our understanding of cellular biology in different species, especially in the understanding of diseases, such as oncological diseases [6].

A large number of reports indicates that abnormal expression of lncRNAs is closely related to various tumors [7,8]. Studies have found that Plnc RNA-1 has an important regulatory role in the proliferation and apoptosis of colorectal cancer cells [9]. However, little has been reported about the expression and biological effects of Plnc RNA-1 in colorectal cancer. Therefore, we intended to study the expression and biological functions of Plnc RNA-1 in human colorectal cancer tissues, and explore its possible molecular mechanisms in order to provide new research ideas for the treatment of colorectal cancer.

Methods

Tissues and cells

A retrospective analysis was performed on 77 colorectal cancer tissues from July 2012 to July 2017 in Weifang People's Hospital. All enrolled patients met the following criteria: a) patients who were first diagnosed with colorectal cancer by pathological examination and had not received any treatment; b) patients without any other complications and in good physical condition. Clinical information before and after treatment was collected and statistical analyses were performed. The study was approved by the Weifang People's Hospital Ethics Committee. All patients and their families signed written informed consent. At the same time, 30 cases of normal colorectal tissue were included, sliced, placed in 1.5 mL EP tube, and stored in the refrigerator at -80°C.

Human normal cell line NCM460 and colorectal cancer cell lines SW116 and LOVO (Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Beijing, China) were cultured in F12 or 1640 medium (Hyclone, South Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 mg/mL) (Hyclone, South Logan, UT, USA). The cells were placed in a constant temperature incubator (5% CO_2, 37°C) and routinely digested and subcultured.

Cell transfection

SW116 and LOVO cells were digested and the cell density was adjusted to 1×10^5 cells/ml with Roswell Park Memorial Institute 1640 (RPMI-1640) culture medium containing 10% FBS. The cells were seeded in 96-well plates (100 µL/well cell suspension) or 6-well plates (2 mL/well cell suspension) with 1×10^5 cells. Cells were transfected when they reached 60-80% confluence with si-NC, pc-DNA-NC, si-Plnc RNA-1 and pc-DNA-Plnc RNA-1. The liposome Lipofectamine 2000 was used for transfection according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA).

RNA extraction and quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

Total RNAs of tissues or cells were isolated by TRIzol reagent (Invitrogen Carlsbad, CA, USA). The RNA concentration was measured by biospectrometer. Complementary DNA (cDNA) was synthesized by reverse transcription according to the instructions of Prime Script RT kit (Perfect Real-time). qRT-PCR reaction system was prepared according to the requirements of SYBR Premix Ex TaqTM (Tli RNaseH Plus). The PCR programs are 95°C for 1 min, 95°C for 30 s, 60°C for 40 s with a total of 40 cycles. In this experiment, 3 replicate wells were set and all samples were tested in triplicate.

Cell counting kit-8 (CCK-8) and colony formation assay

The viability and proliferation ability of the cells were measured by CCK8 and colony formation assay (Dojindo, Kumamoto, Japan). After transient transfection for 6 hrs, the cells were collected and the density was adjusted to 4×10^4/mL. The cells were seeded in 96-well plates (80 µL/well) and 6 replicate wells were set in each group. CCK-8 mixtures containing serum-free medium (1:10) were added to 96-well plates (110 mL/well) at 0, 6, 24, 48, 72 and 96 hrs, respectively. The optical density (OD) at a wavelength of 450 nm was measured with a microplate reader and the growth curve was plotted. For colony formation assay, transfected cells were seeded in 6-well plates (1000 cells/well) and the medium was changed after 3 days. After 14 days, cells were fixed in formaldehyde and stained with Crystal Violet. Then cells were counted and photographed.

Flow cytometry

The cell cycle can be divided into G1/G0, S and G2/M phase according to the intracellular DNA content detected by flow cytometry and propidium iodide (PI) staining. After transfection for 24 hrs, cells were collected and the supernatant was removed by centrifugation. After washing twice with phosphate buffered saline (PBS), the cells were resuspended in 1×Binding buffer and stained with 300-500 µL of the labeling solution. Then, the cell cycle was detected on a FACS cytometer.

Western blot

Total proteins were extracted by a radioimmunoprecipitation assay (RIPA) kit (Beyotime, Beijing, China). Protein concentration was detected using the bicinchoninic acid (BCA) kit (Beyotime, Beijing, China). Then, the proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a 0.22 μm polyvinylidene fluoride (PVDF) membrane (Roche, Basel, Switzerland). The membrane was then blocked with 5% skim milk and incubated with primary antibodies p-GSK3β, p-Rb, and CyclinD1 (dilution 1:1000, CST, Danvers, MA, USA) at 4°C overnight. Glyceraldheyde 3-phosphate dehydrogenase (GAPDH) was used as internal control. After washing three times with TBST (5 min/time), the membrane was incubated with the second antibody (diluted 1:1000, CST, Danvers, MA, USA) at room temperature for 2 hrs, and then the membrane was washed three times with TBST (10 min/time). Protein bands were determined by image analysis system with enhanced chemiluminescence (ECL) imaging (Thermo Fisher Scientific, Waltham, MA, USA).

Statistics

SPSS 19.0 software was used for statistical analyses (IBM, Armonk, NY, USA). The normal distribution of quantitative data was expressed as mean±standard deviation. ROC curve was used to show the correlation of Plnc RNA-1 expression and colorectal cancer diagnosis. The t-test was adopted to compare differences between groups. The skewed distribution was taken as the median (quartile), and the Wilcoxon rank test was used for the non-parametric total rank of grade data. Differences were statistically significant at $p<0.05$.

Results

High expression of Plnc RNA-1 in colorectal cancer

Real-time PCR results showed that Plnc RNA-1 expression was significantly higher in 77 colorectal cancer tissues compared with the control group ($p<0.05$; Figure 1A). In addition, the expression level of Plnc RNA-1 in the infiltrating T3+T4 patients was significantly higher than that in the infiltrating T1+T2 group ($p<0.05$; Figure 1B). At the same time, the expression level of Plnc RNA-1 in stages III+IV patients was higher than that in stages I+II patients ($p<0.05$; Figure 1C). A 10-year follow-up survival analysis revealed that colorectal cancer patients with high expression of Plnc

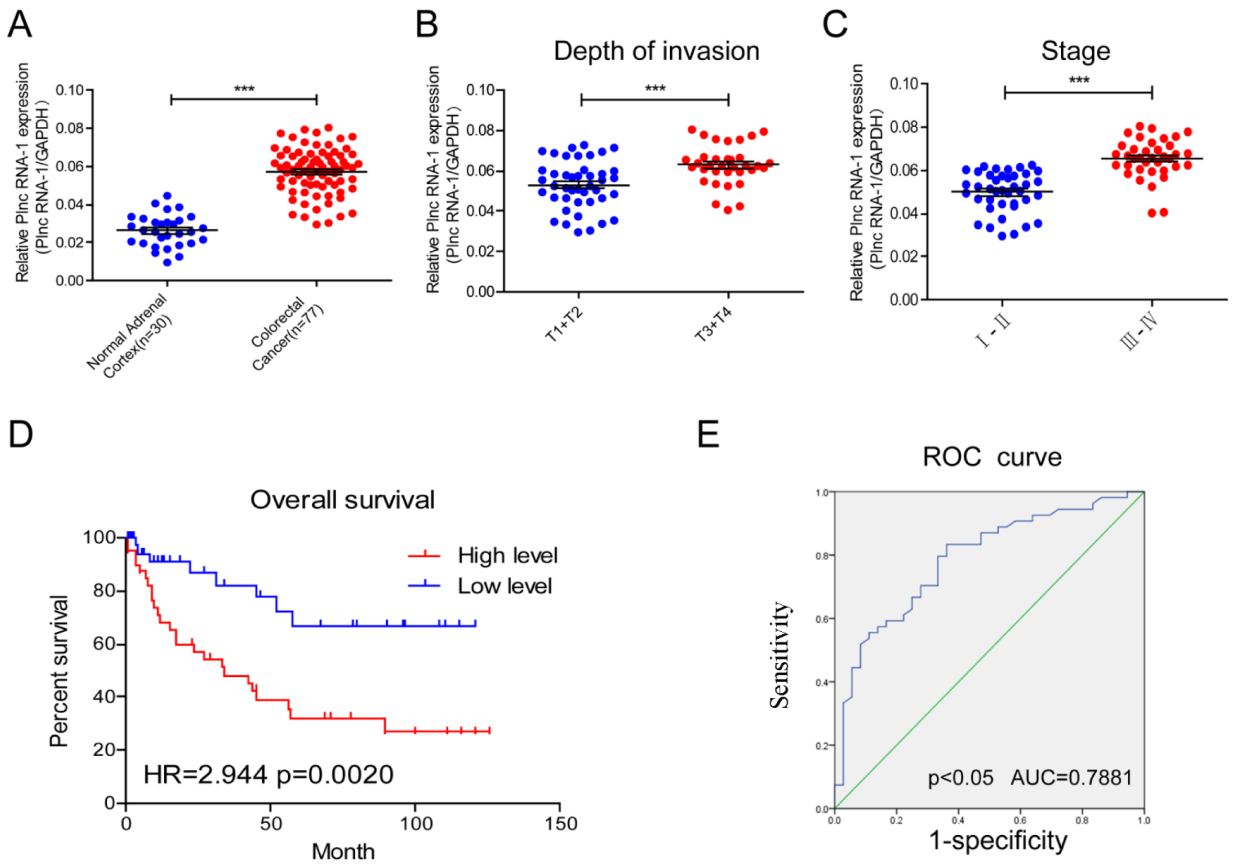

Figure 1. High expression of Plnc RNA-1 in colorectal cancer. **A:** Expression of Plnc RNA-1 in 77 colorectal cancer patients was significantly higher than in 30 controls. **B:** Expression of Plnc RNA-1 in infiltrating T3+T4 patients was higher than in infiltrating T1+T2. **C:** Expression of Plnc RNA-1 in stage III+IV patients was higher than stage I+II. **D:** Overall survival of Plnc RNA-1 in high expression group was significantly lower than in low expression group. **E:** ROC curve between Plnc RNA-1 expression and diagnostic sensitivity of colorectal cancer. ***p<0.001.

Table 1. Correlation between Plnc RNA-1 expression and clinicopathological characteristics of colorectal cancer

Clinicopathological characteristics	*Number of cases*	*Plnc RNA-1 expression*		*p value*
		Low (n=38)	*High (n=39)*	
Age, years				0.9056
≤50	40	20	20	
>50	37	18	19	
Gender				0.4271
Male	29	16	13	
Female	48	22	26	
Tumor size, cm				0.0087
≤3	55	32	23	
>3	15	3	12	
TNM stage				0.0034
I~II	46	29	17	
III~IV	31	9	22	
Lymph node metastasis				0.0502
Absent	62	34	28	
Present	15	4	11	

RNA-1 levels had lower survival rate than the low expression group (HR=2.944, p=0.002; Figure 1D). The ROC curve showed that the expression of Plnc RNA-1 was highly correlated to the diagnosis of colorectal cancer (AUC=0.7881, p<0.05; Figure 1E). Besides, higher expression of Plnc RNA-1 was connected with advanced stage and larger tumor size (Table 1). These results indicated that Plnc RNA-1 was highly expressed in colorectal cancer patients, and there was a negative correlation between Plnc RNA-1 expression and prognosis.

High expression of Plnc RNA-1 promoted proliferation of colorectal cancer cells

Real-time PCR results showed that Plnc RNA-1 was lowly expressed in normal cell line NCM460 and highly expressed in colorectal cancer cell lines including SW116 and LOVO, with the highest expression in LOVO cells (p<0.05; Figure 2A). In the LOVO cell line, Plnc RNA-1 expression was significantly decreased (p<0.05) after transfection with the Plnc RNA-1 interference sequence (Figure 2B). In the SW116 cell line, after transfection with the Plnc RNA-1 overexpression plasmid, the level of Plnc RNA-1 was significantly increased (p<0.05; Figure 2C). In the LOVO cells, knocking down Plnc RNA-1 significantly decreased the cell viability (p<0.05; Figure 2D), and the cell proliferative capacity was significantly decreased (p<0.05; Figure 2E). In the SW116 cells, cell viability was significantly increased after overexpression of Plnc RNA-1 (Figure 2F), and the proliferation ability was significantly enhanced (p<0.05; Figure 2G). The above results indicated that high expression of Plnc RNA-1 could promote the proliferation of colorectal cancer cells and enhance cell activity.

Plnc RNA-1 participates in the development of colorectal cancer by regulating the cell cycle

Next, we used flow cytometry to detect the cell cycle. We found that knocking down Plnc RNA-1 promoted the cell cycle and that the cell number in G0/G1 phase increased significantly in the LOVO cell line (p<0.05; Figure 3A). In the SW116 cell line, after overexpression of Plnc RNA-1, the cell cycle was arrested and the number of G0/G1 phase cells was significantly reduced (p<0.05; Figure 3B). Besides, the expression of the cell cycle-related proteins p-GSK3β, p-Rb, and CyclinD1 was significantly reduced after knocking down Plnc RNA-1 in the LOVO cell line (Figure 3C). However, the expression of p-GSK3β, p-Rb, and CyclinD1 was significantly increased after Plnc RNA-1 overexpression in the SW116 cell line (Figure 3D). These results demonstrated that Plnc RNA-1 participated in the development of colorectal cancer by regulating the cell cycle.

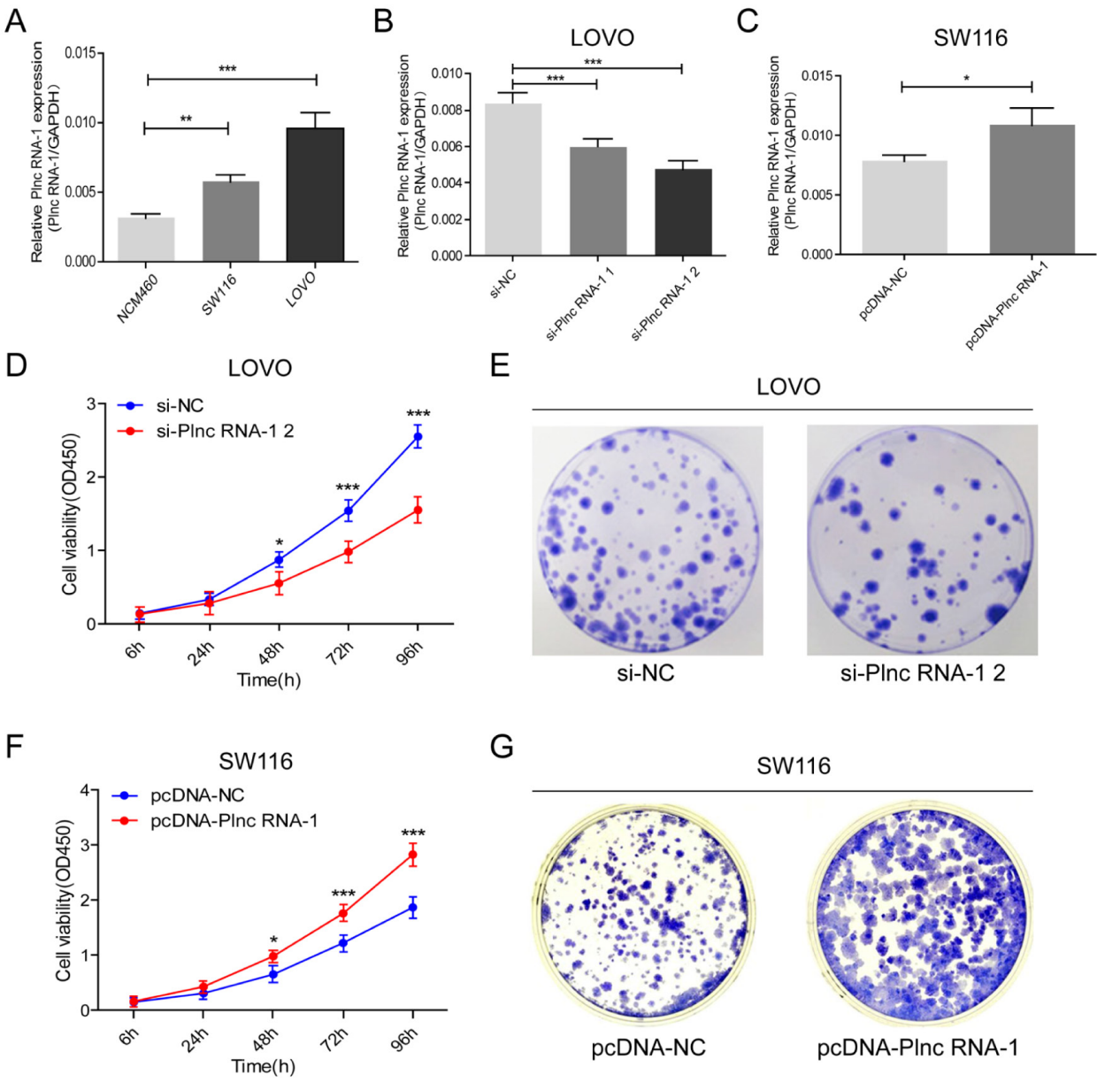

Figure 2. High expression of Plnc RNA-1 promotes colorectal cancer cells proliferation. **A:** Expression level of Plnc RNA-1 in NCM460 and SW116, LOVO. **B:** Expression level of siPlnc RNA-1 transfection in LOVO. **C:** Plnc RNA-1 expression levels in SW116 after overexpression of Plnc RNA-1. **D:** Knockdown Plnc RNA-1 expression decreased cell viability in LOVO. **E:** Knockdown Plnc RNA-1 expression reduced the cell's proliferative capacity in LOVO. **F:** Cell viability increased after Plnc RNA-1 overexpression in SW116. **G:** Knockdown of Plnc RNA-1 expression resulted in increased cell proliferation in SW116. *p<0.05, **p<0.01, ***p<0.001.

Discussion

Colorectal cancer is a common malignancy with a high incidence in China. In recent years, its incidence rate is constantly increasing, while metastasis is the main cause of death [10,11]. Although many oncogenes, tumor suppressor genes and cell cycle-related signaling pathways have been discovered in recent years, the pathogenesis of colorectal cancer remains unclear [12].

Long non-coding RNAs (lncRNAs) are a type of RNAs longer than 200 nt and cannot encode proteins [13]. In recent years, studies have found that lncRNAs play an important role in the regulation of angiogenesis in various tumors, such as breast cancer, liver cancer, cholangiocarcinoma and gastric cancer [14-16]. Research found that Plnc RNA-1 was upregulated in colorectal tissue. Plnc RNA-1 interference in colorectal cancer cells inhibited tumor growth, promoted apoptosis, and inhibited the androgen receptor (AR) signal pathway. It has been proved that Plnc RNA-1 can act as an oncogene which regulates tumor growth [17]. Recent studies have also found that multiple ln cRNAs

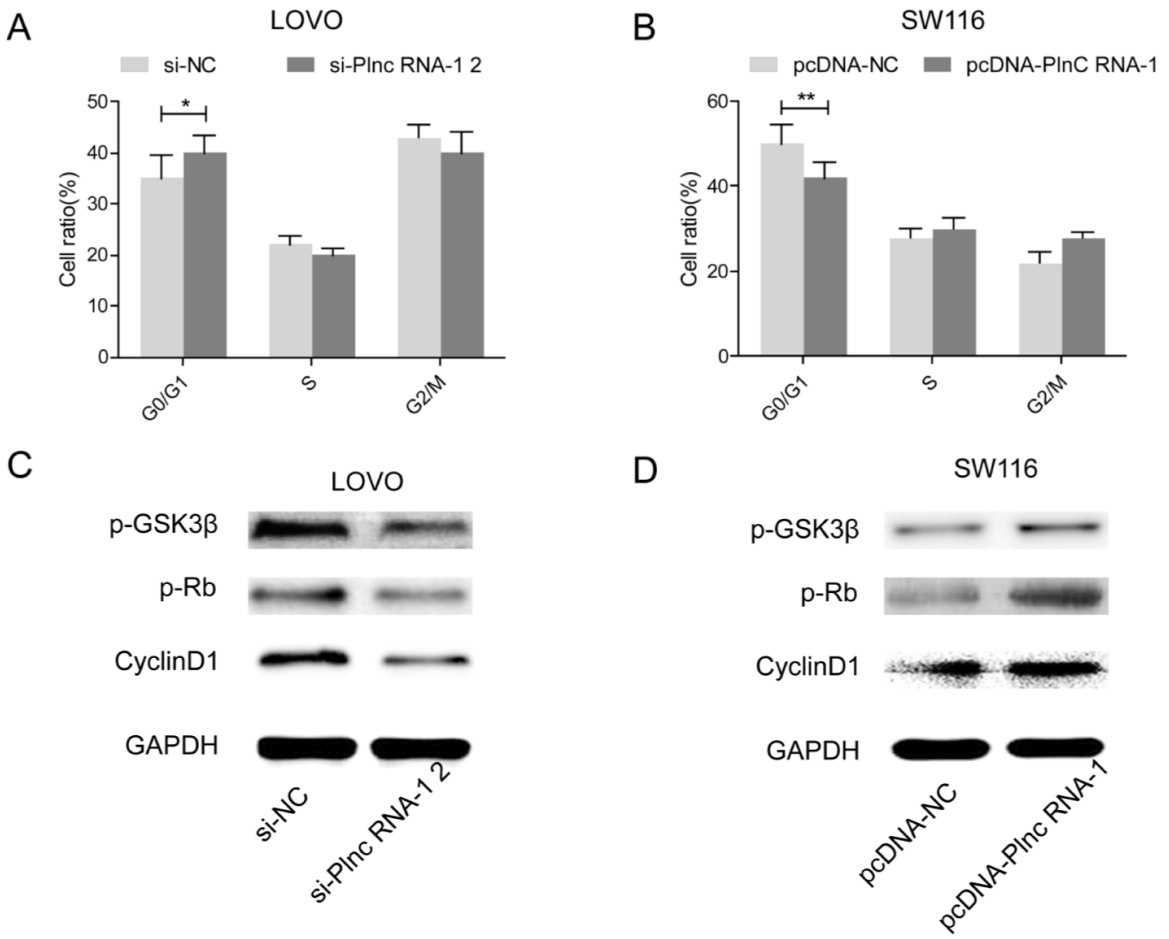

Figure 3. Plnc RNA-1 participates in colorectal cancer development by regulating the cell cycle. **A:** In LOVO, Plnc RNA-1 knockdown promotes cell cycle. **B:** In SW116, the cell cycle was arrested after Plnc RNA-1 overexpression. **C:** In LOVO, after Plnc RNA-1 knockdown, the expression of the cell cycle-related proteins p-GSK3β, p-Rb, and CyclinD1 was reduced. **D:** In SW116, after Plnc RNA-1 overexpression, the expression of the cell cycle-related proteins p-GSK3β, p-Rb, and CyclinD1 was increased. *p<0.05, **p<0.01.

are differently expressed between colorectal cancer cells and normal colorectal cells [18,19]. Analyzing the expression pattern of different lncRNAs in normal colorectal cells and colorectal cancer cells can provide more accurate conclusions for the diagnosis of colorectal cancer [20-22]. In this study, we first found that Plnc RNA-1 was highly expressed in colorectal cancer cells and that Plnc RNA-1 intervention inhibited tumor cell proliferation and promoted tumor cell apoptosis.

The present study also showed that the expression of Plnc RNA-1 was higher in infiltrating T3+T4 patients than in T1+T2 patients. Stages III+IV patients had higher Plnc RNA-1 expression levels than those in stages I+II. Survival of patients with high expression of Plnc RNA-1 was significantly lower than in patients with low expression. The expression of Plnc RNA-1 was positively correlated with the diagnostic sensitivity of colorectal cancer, suggesting that Plnc RNA-1 can promote the development of colorectal cancer and affect prognosis. As a key gene regulatory factor, Plnc RNA-1 overexpression increased the cell viability and proliferation significantly in the SW116 cell line. In the LOVO cell line, cell viability and proliferation displayed an obvious reduction after knockdown of Plnc RNA-1. Cell cycle evaluated by flow cytometry showed that in the LOVO cell line, knocking down of Plnc RNA-1 promoted the cell cycle and the cell cycle-related proteins, such as p-GSK3β, p-Rb, while CyclinD1 was decreased. In SW116 cell line, overexpression of Plnc RNA-1 led to cell cycle arrest and the expression of cell cycle-related proteins was increased. All these suggested that Plnc RNA-1 promoted the progression of colorectal cancer by regulating the cell cycle.

In summary, Plnc RNA-1 upregulates the cell cycle progression and promotes the development of colorectal cancer. Therefore, inhibiting Plnc RNA-1 expression can reverse the development of colorectal cancer partially, suggesting that Plnc RNA-1 plays an important role in promoting the

occurrence and development of colorectal cancer, which provides a new basis theory for the gene therapy of colorectal cancer.

Conclusions

The expression of Plnc RNA-1 is significantly upregulated in colorectal cancer cells. Plnc RNA-1 participates in colorectal cancer development by regulating the cell cycle and provides a new theoretical basis and a new target for the treatment of colorectal cancer.

Conflict of interests

The authors declare no conflict of interests.

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