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CASE REPORT

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ABOUT COVER

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ORIGINAL ARTICLE

Basic Study

Long noncoding RNA RP4 functions as a competing endogenous RNA through miR-7-5p sponge activity in colorectal cancer

Mu-Lin Liu, Qiao Zhang, Xiao Yuan, Long Jin, Li-Li Wang, Tao-Tao Fang, Wen-Bin Wang

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Author contributions: Liu ML, Zhang Q and Yuan X contributed equally to this work; Liu ML and Zhang Q conceived the study and participated in its design and coordination; Yuan X drafted and revised the manuscript; Jin L helped with the statistical analysis; Wang LL and Fang TT performed the experiments; Wang WB conceived the study and revised the manuscript; all authors read and approved the final manuscript.

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Abstract

AIM

To investigate the role of long noncoding RNA (lncRNA) RP4 in colorectal cancer.

METHODS
Lentivirus-mediated lncRNA RP4 overexpression and knockdown were performed in the colorectal cancer cell line SW480. Cell proliferation, tumor growth, and early apoptosis were evaluated by a cell counting kit-8 assay, an *in vivo* xenograft tumor model, and annexin V/propidium iodide staining, respectively. Analysis of the lncRNA RP4 mechanism involved assessment of the association of its expression with miR-7-5p and the *SH3GLB1* gene. Western blot analysis was also performed to assess the effect of lncRNA RP4 on the autophagy-mediated cell death pathway and phosphatidylinositol-3-kinase (PI3K)/Akt signaling.

RESULTS
Cell proliferation, tumor growth, and early apoptosis in SW480 cells were negatively regulated by lncRNA RP4. Functional experiments indicated that lncRNA RP4 directly upregulated *SH3GLB1* expression by acting as a competing endogenous RNA (ceRNA) for miR-7-5p. This interaction led to activation of the autophagy-mediated cell death pathway and de-repression of PI3K and Akt phosphorylation in colorectal cancer cells *in vivo*.

CONCLUSION
Our results demonstrated that lncRNA RP4 is a ceRNA that plays an important role in the pathogenesis of colorectal cancer, and could be a potential therapeutic target for colorectal cancer treatment.

Key words: Colorectal cancer; Long noncoding RNA RP4; SH3GLB1; miR-7-5p; Competing endogenous RNA

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Core tip: In the present study, we investigated the role of long noncoding RNA (lncRNA) RP4 in colorectal cancer using an *in vivo* cell model and an *in vivo* xenograft model. Mechanistic analysis suggested that lncRNA RP4 functions in colorectal cancer pathogenesis as a competing endogenous RNA that regulates SH3GLB1 expression by acting as a sponge for miR-7-5p. It could also serve as a potential therapeutic target for colorectal cancer treatment.

Liu ML, Zhang Q, Yuan X, Jin L, Wang LL, Fang TT, Wang WB. Long noncoding RNA RP4 functions as a competing endogenous RNA through miR-7-5p sponge activity in colorectal cancer. *World J Gastroenterol* 2018; 24(9): 1004-1012 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i9/1004.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i9.1004

INTRODUCTION

Colorectal cancer is the fourth most common cancer and the fifth most common cause of cancer-related death in China, with an estimated 331300 newly diagnosed patients and 159300 deaths in 2012[1]. Surgical resection followed by adjuvant chemotherapy is the most commonly used strategy for colorectal cancer management. However, although the overall 5-year survival rate of colorectal cancer has improved to 65%, the 5-year survival rate was only 15% in patients presenting with distant metastasis[2], reflecting the poor treatment response in some patients. Therefore, it is necessary to identify effective therapeutic targets to improve treatment and prognosis.

Long noncoding RNAs (lncRNAs), > 200 nucleotides in length, are a recently discovered novel class of genes with regulatory functions but lacking protein-coding ability. Several studies have identified important roles for lncRNAs in a wide range of cellular processes, including X chromosome inactivation, splicing, imprinting, epigenetic control, and gene transcription regulation[3-5]. Moreover, the dysregulated expression of lncRNAs is present in various human diseases, especially in cancers including breast cancer, lung cancer, gastric cancer, and colorectal cancer[6-8]. Indeed, several recent pieces of evidence suggest that lncRNAs are involved in the development and progression of human colorectal cancer and may serve as novel therapeutic targets[9-11]. However, the role of lncRNAs in colorectal cancer is largely unknown.

The dysregulation of lncRNA RP4 has previously been shown by expression profile analysis of a transcriptome microarray. Therefore, the present study investigated the role of lncRNA RP4 in colorectal cancer using an *in vitro* cell model and an *in vivo* xenograft model. Mechanistic analysis suggested that lncRNA RP4 functions in colorectal cancer pathogenesis as a competing endogenous RNA (ceRNA) that regulates *SH3GLB1* expression by acting as a sponge for the microRNA (miRNA) miR-7-5p. It could also serve as a potential therapeutic target for colorectal cancer treatment.

MATERIALS AND METHODS

Ethics statement

This study was conducted in accordance with the ethical standards, the Declaration of Helsinki, and national and international guidelines, and was approved by the authors' institutional review board, which adheres to generally accepted international guidelines for animal experimentation.

Cell culture

The human colorectal cancer cell line SW480 was obtained from the American Type Culture Collection. Cells were maintained as monolayers in cell culture flasks with RPMI1640 medium containing 10% (v/v) fetal bovine serum and 1% antibiotics. They were cultured at 37 ℃ in a humidified atmosphere with 5% CO_2. All cell culture media and additives were purchased from Invitrogen (CA, United States).

Lentiviral short hairpin (sh)RNA particles

Recombinant lentiviral particles expressing lncRNA RP4

Table 1 Sequences of the primers used

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
ENST00000565575	ATCCGTTCCAAATCCTGTCGT	TTCAAGCAGAGGCTGTATCGTG
SH3GLB1	CGCTGTCTGAATGACTTTGT	CCTTTCTGCTGCCACTACAC
β-actin	GTGGCCGAGGACTTTGATTG	CCTGTAACAACGCATCTCATATT

or lncRNA RP4 small interfering (si)RNA were obtained from GenePharm Co., Ltd. (Shanghai, China). Cells were grown to approximately 40% confluence and infected with lentiviral particles in complete medium for 48 h. To increase the infection efficiency, cells were co-treated with the cationic polymer polybrene (8 μg/mL in water). Neither shRNA nor polybrene affected cell viability. siRNA and shRNA had no off-target effects, and did not affect cell adherence, shape, or viability at the indicated multiplicity of infection.

Real-time quantitative reverse transcription polymerase chain reaction

Total RNA was extracted from SW480 cells using TRIzol reagent (Invitrogen). RT-PCR was carried out using a One Step SYBR PrimeScript RT-PCR kit (Takara, Dalian, China) and an iQ5 Real-time PCR Detection system (Bio-Rad, Hercules, CA, United States) for evaluation of the expression of lncRNA RP4. The miRNA miR-7-5p was obtained using the PureLink miRNA Isolation Kit (Invitrogen), and the quantification of miRNA expression was performed with a TaqMan MicroRNA Assay Kit (Applied Biosystems, Foster City, CA, United States). The expression of β-actin and *U6* snRNA genes was assessed simultaneously in all samples as an internal control for lncRNA/mRNA and miRNA expression, respectively. Relative gene expression was determined by the $2^{-\Delta\Delta CT}$ method[12]. Oligonucleotide primers specific for lncRNA RP4, *SH3GLB1*, and β-actin are listed in Table 1.

Western blot analysis

Cells were lysed in RIPA buffer, centrifuged at high speed, and then underwent protein quantification using a bicinchoninic acid assay. Cellular proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. After blocking, the membranes were incubated with anti-total- or -phosphor-PI3K, phospho-Akt, LC3A/B, Bax, and caspase 3 monoclonal primary antibodies (Cell Signaling Technology, Cambridge, MA, United States). β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, United States) was used as the loading control. Appropriate horseradish peroxidase-conjugated secondary antibodies were applied to detect labeled proteins. The protein bands were developed with SuperSignal Ultra Chemiluminescent Substrate (Pierce, Rockford, IL, United States) on X-ray films (Kodak, Tokyo, Japan).

Cell proliferation

SW480 cells (3×10^3 cells) were seeded in 96-well plates in complete medium and infected with lncRNA RP4, lncRNA RP4 siRNA, or control lentivirus particles. Two days later, cell proliferation was evaluated by the cell counting kit-8 method according to the manufacturer's instructions using a microplate reader (Molecular Devices, Sunnyvale, CA, United States) to measure the absorbance.

Nude mouse model of ectopic tumors

Athymic nude (nu/nu) mice at 6 wk old were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. Tumors were generated by the subcutaneous injection of 2×10^6 SW480 cells infected with lncRNA RP4, lncRNA RP4 siRNA, or control lentivirus particles and suspended in 50 μL of PBS into the dorsal region near the thigh. Mice were then weighed and assessed for tumor size every 7 wk by measuring the tumor length and width.

Cell apoptosis analysis

SW480 cells (3×10^5 cells) were seeded in 6-well plates in complete medium and infected with lncRNA RP4, lncRNA RP4 siRNA, or control lentivirus particles. Two days later, cell proliferation was evaluated by flow cytometry (FACScalibur; BD Biosciences, CA, United States) after annexin V/propidium iodide staining (Beyotime institution, Nantong, China).

Statistical analysis

All statistical analyses were carried out using SPSS v18 software (SPSS, Chicago, IL, United States). Data are presented as the mean ± SD. The Student's *t*-test or one-way analysis of variance were used to examine differences between two or multiple groups. Correlation analyses of the expression levels of lncRNA RP4, *SH3GLB1*, and miR-7-5p were performed using Pearson's correlation coefficient. A P-value < 0.05 was considered statistically significant.

RESULTS

lncRNA RP4 regulates proliferation, tumor growth, and early apoptosis in colorectal cancer cells

To investigate the role of lncRNA RP4 in the pathogenesis of colorectal cancer, we performed lentivirus-mediated overexpression and knockdown. As shown in Figure 1A, SW480 cell proliferation was negatively regulated by lncRNA RP4, while early apoptosis was positively regulated by lncRNA RP4 (Figure 1C and D). These results suggested that lncRNA RP4 exerts a negative regulatory role in colorectal cancer cell

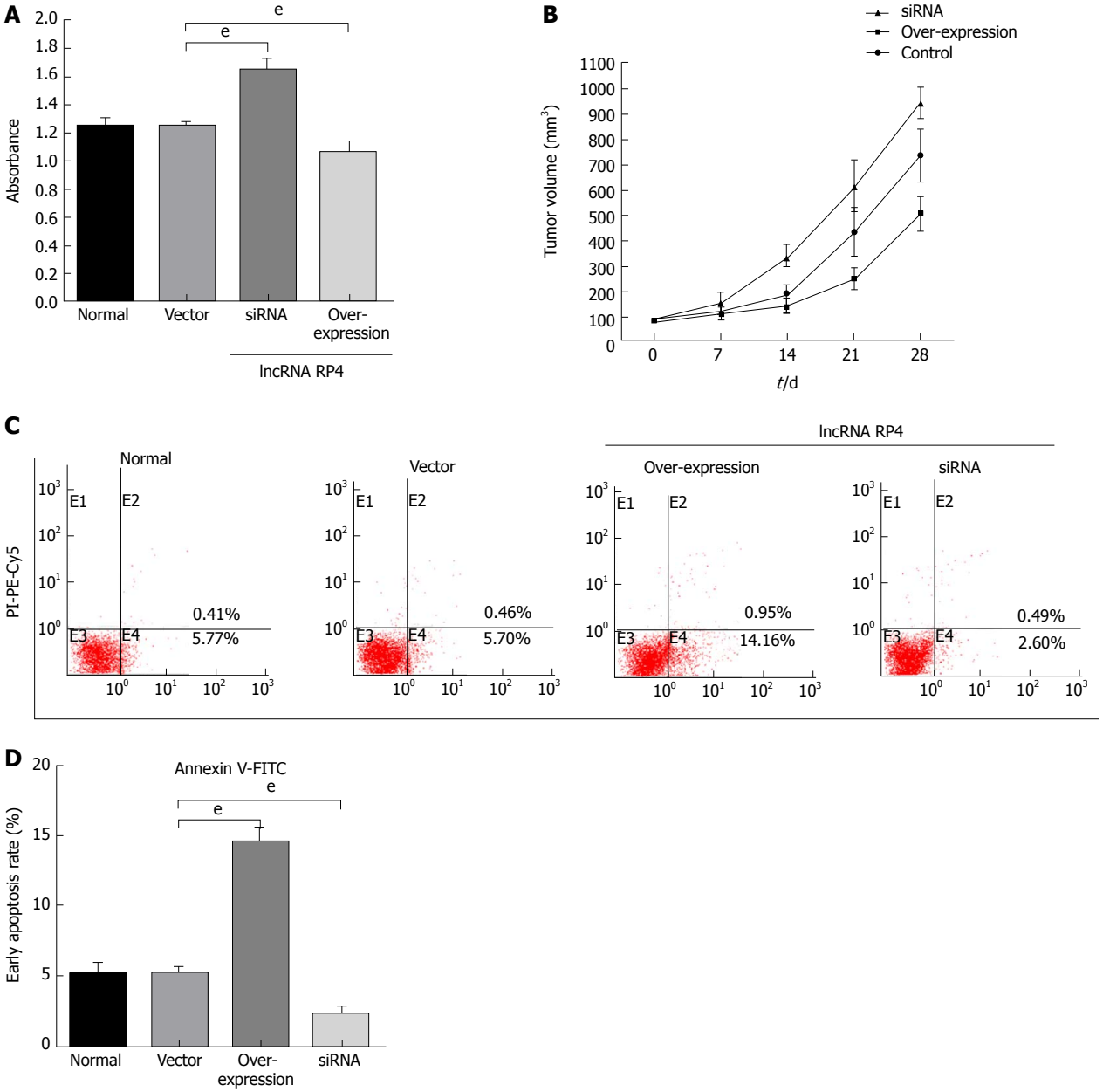

Figure 1 lncRNA RP4 regulates proliferation, tumor growth, and early apoptosis in colorectal cancer cells. Lentivirus-mediated lncRNA RP4 overexpression and knockdown were performed in the colorectal cancer line SW480, and cell proliferation, tumor growth, and early apoptosis were examined. A: Cell proliferation was examined by the CCK-8 assay. lncRNA RP4 overexpression and knockdown were shown to decrease and increase cell proliferation, respectively. B: Tumor growth was evaluated by tumor volume change. lncRNA RP4 overexpression and knockdown were shown to significantly decrease and increase tumor volume, respectively, at weeks 14, 21, and 28. C: Flow cytometry assessment of early apoptosis. lncRNA RP4 overexpression and knockdown increased and decreased early apoptosis, respectively, in colorectal cancer. D: Early apoptosis quantification. $^{e}P < 0.001$ for between-group comparisons.

proliferation and a positive regulatory role in early apoptosis of colorectal cancer cells.

lncRNA RP4 inhibits the growth of colorectal cancer on mice

Compared with the control group, colorectal cancer with lncRNA RP4 siRNA showed a bigger volume, while there was a smaller volume in the group with lncRNA RP4 overexpression (Figure 1B). Consistent with the results in cell line, the results *in vivo* also suggested that lncRNA RP4 plays an inhibitory role in colorectal cell growth.

lncRNA RP4 inhibits the growth of colorectal cancer cells by regulating SH3GLB1

To explore the mechanism of lncRNA RP4-mediated effects in colorectal cancer cells, we examined *SH3GLB1* expression in SW480 cells following lncRNA RP4 overexpression and knockdown. lncRNA RP4 was found to positively regulate *SH3GLB1* expression, and correlation analyses further confirmed the existence of a significant correlation between lncRNA RP4 and

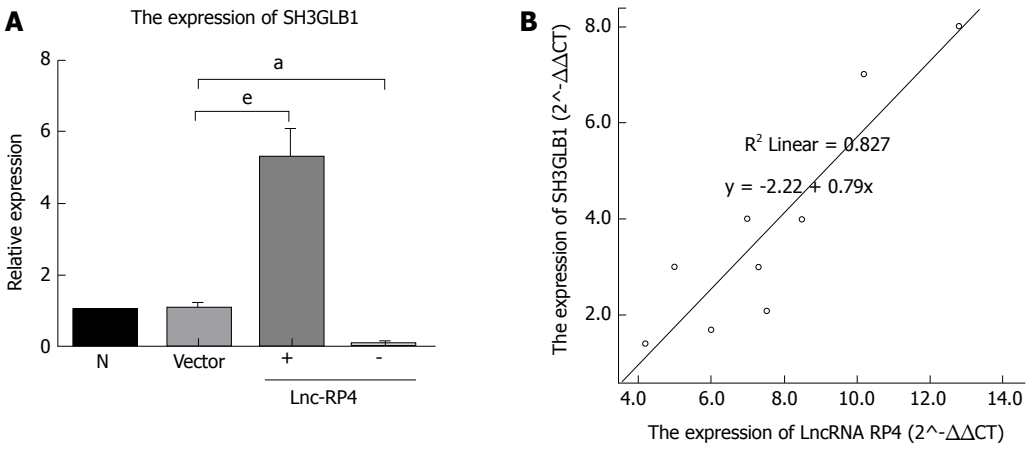

Figure 2 lncRNA RP4 affects the expression of *SH3GLB1* in colorectal cancer cells. Lentivirus-mediated lncRNA RP4 overexpression and knockdown were performed in *SW480* cells, and *SH3GLB1* expression was evaluated by real-time quantitative PCR, followed by association analyses between *SH3GLB1* and lncRNA RP4 levels. A: LncRNA RP4 overexpression and knockdown, respectively, increased and decreased *SH3GLB1* expression in SW480 cells. B: Correlation analyses revealed a linear association between the expression of *SH3GLB1* and lncRNA RP4, with an r^2 value of 0.827. $^aP < 0.05$ and $^eP < 0.001$ for between-group comparisons.

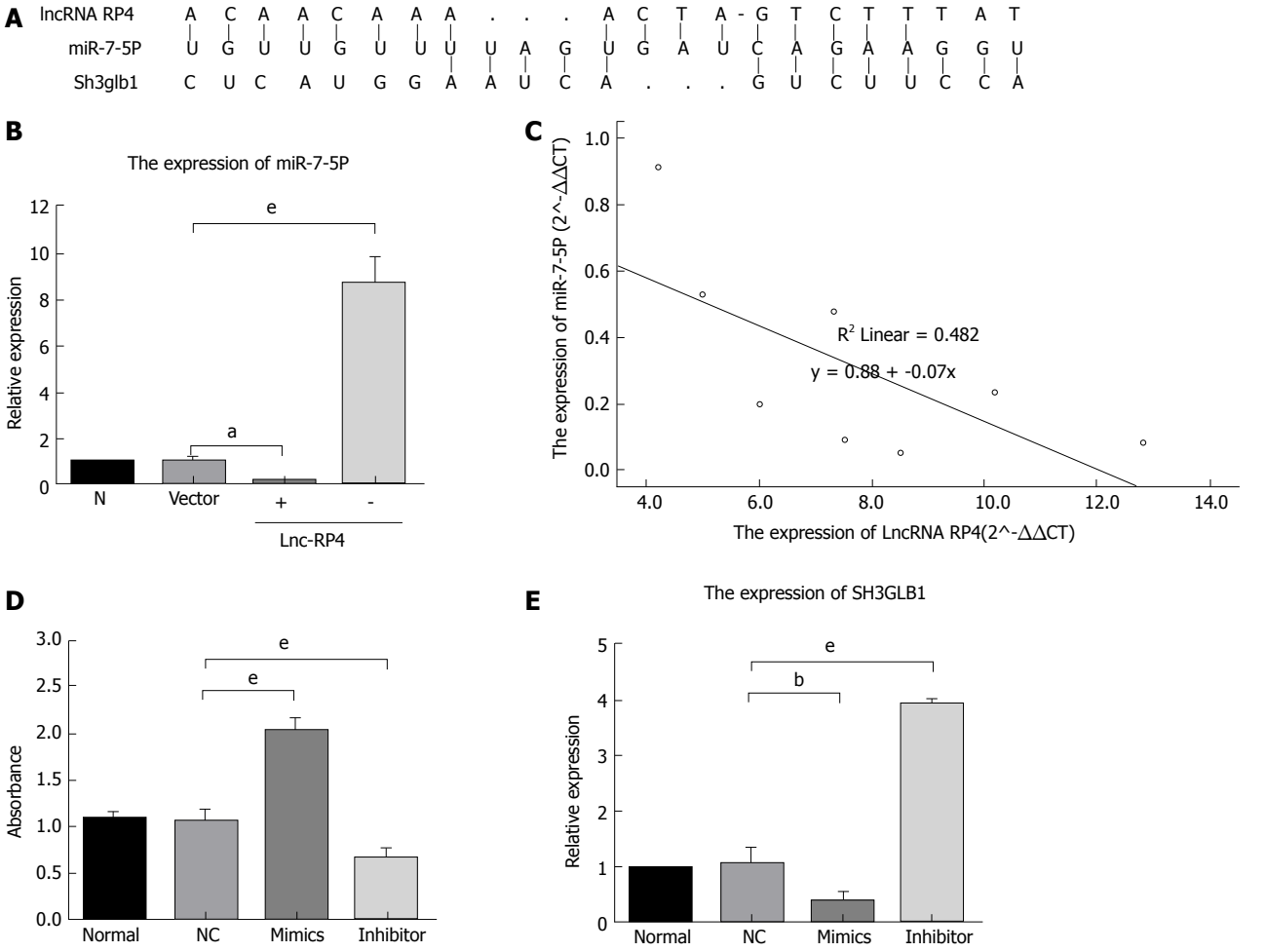

Figure 3 lncRNA RP4 functions as an miR-7-5p decoy in colorectal cancer cells. A: The predicted miR-7-5p binding sites on the *SH3GLB1* and lncRNA RP4 transcript. B: LncRNA RP4 overexpression and knockdown, respectively, decreased and increased the expression of miR-7-5p in SW480 cells. C: Correlation analyses revealed a linear association between the expression of lncRNA RP4 and miR-7-5p, with an r^2 value of 0.482. D: *SW480* cells were transfected with an miR-7-5p mimic and inhibitor, and cell proliferation was evaluated by the CCK-8 assay. miR-7-5p overexpression and knockdown increased and decreased cell proliferation, respectively. E. Real-time quantitative PCR showed that miR-7-5p overexpression and knockdown, respectively, decreased and increased *SH3GLB1* expression level in SW480 colorectal cancer cells. $^aP < 0.05$, $^bP < 0.01$, and $^eP < 0.001$ for between-group comparisons.

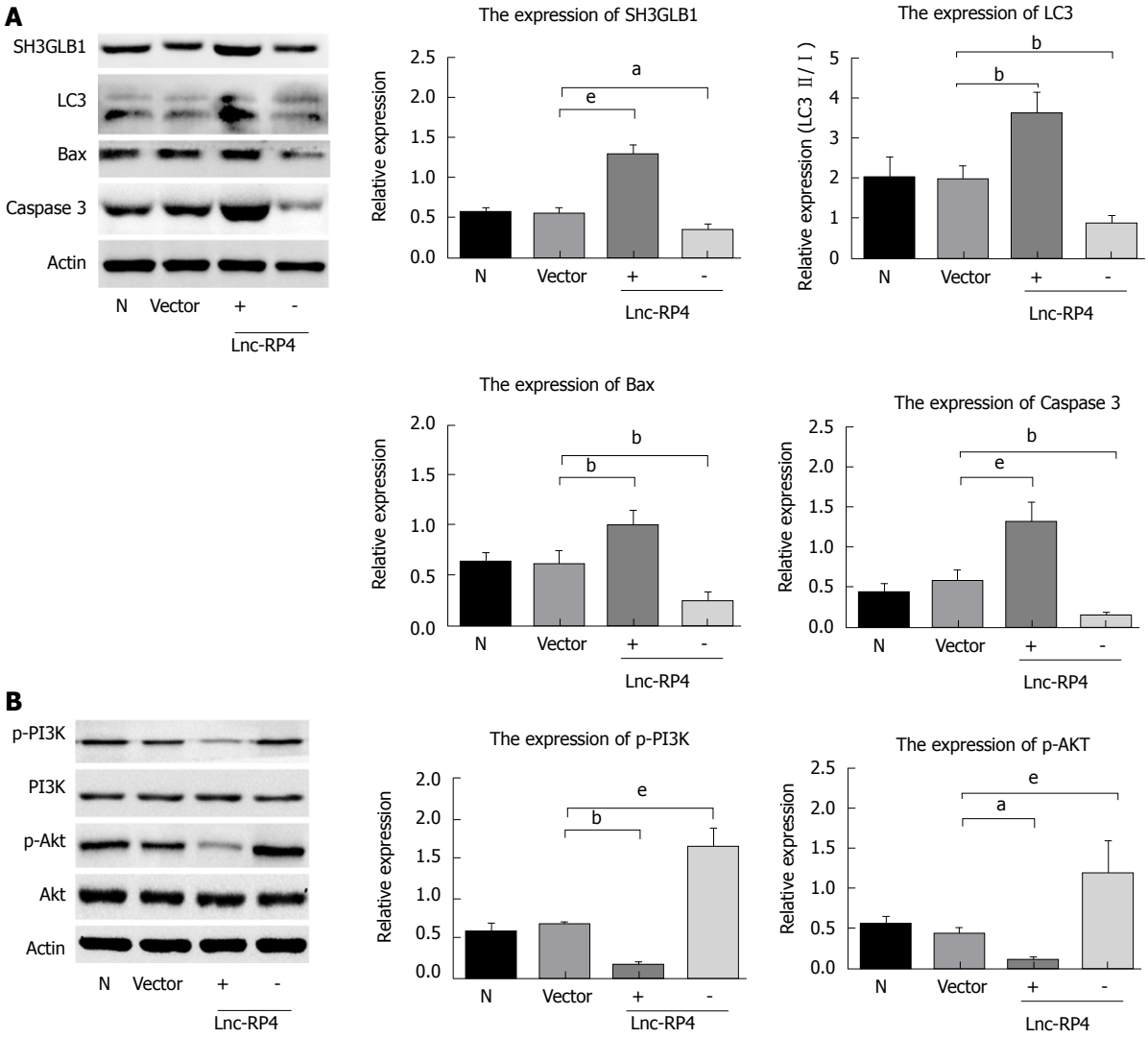

Figure 4 Involvement of the autophagy-mediated cell death pathway and PI3K/Akt signaling pathway in lncRNA RP4-mediated effects in colorectal cancer cells. A: LncRNA RP4 overexpression and knockdown, respectively, decreased and increased expression of the autophagy marker LC3, and apoptosis-related proteins Bax and caspase 3 in SW480 cells, suggesting that it positively regulates autophagy-mediated cell death in colorectal cancer cells. B: LncRNA RP4 overexpression and knockdown, respectively, decreased and increased PI3K and Akt phosphorylation in SW480 cells, indicating that it negatively regulates PI3K/Akt in colorectal cancer cells. C: Schematic of lncRNA RP4 functioning as a decoy by competitively binding miR-7-5p, upregulating the specific repressor SH3GLB1, activating autophagy-mediated cell death, and inhibiting the PI3K/Akt signaling pathway, thereby suppressing colorectal carcinogenesis. $^{a}P < 0.05$, $^{b}P < 0.01$, and $^{e}P < 0.001$ for between-group comparisons.

SH3GLB1 expression (Figure 2).

lncRNA RP4 functions as an miR-7-5p decoy in the regulation of SH3GLB1

Because no direct interaction exists between lncRNA RP4 and *SH3GLB1*, we further analyzed the potential functional mechanism by the introduction of miRNA. lncRNAs were recently reported to act as decoys that sequester miRNAs and prevent them from binding to targets, hence modulating many functional mRNA targets through translation. Bioinformatics analysis (webserver lnCeDB; http://gyanxet-beta.com/lncedb/) predicted potential interactions between lncRNA RP4 and miR-7-5p (Figure 3A), which was confirmed by correlation analysis (Figure 3B and C). We also observed a positive regulatory effect of miR-7-5p on cell proliferation *via* the negative regulation of *SH3GLB1* (Figure 3D and E). These results suggested that lncRNA RP4 functions as an miR-7-5p decoy in colorectal cancer cells.

Involvement of the autophagy-mediated cell death pathway and PI3K/Akt signaling pathway in lncRNA-RP4 mediated effects in colorectal cancer cells

According to previous findings[13,14], autophagy-mediated cell death is involved in the early apoptosis of cancer, while the PI3K/Akt signaling pathway plays a role in cancer cell proliferation and growth[15,16]. Analysis of the effects of lncRNA-RP4 on intracellular signaling revealed that lncRNA-RP4 overexpression and knockdown, respectively, upregulated and downregulated expression levels of the autophagy marker LC3 and apoptosis-related molecules Bax and caspase 3 (Figure 4A). We also observed the negative regulation of PI3K and Akt phosphorylation by lncRNA-RP4 in colorectal cancer cells (Figure 4B). Taken together, we propose a schematic whereby lncRNA RP4 functions as a decoy that competitively binds miR-7-5p, upregulating the specific repressor *SH3GLB1*, activating autophagy-mediated cell death, and inhibiting PI3K/Akt signaling, thereby suppressing colorectal carcinogenesis (Figure 4C).

DISCUSSION

Noncoding regions account for more than 90% of the entire human genome, and are thought to play a critical role in the regulation of physiological function given that only 9% of human genes are protein-coding. As a representative of noncoding regions, approximately 18% of lncRNAs are associated with human tumors and have been shown to act as major contributors in the development and progression of human cancers[17]. Multiple mechanisms have been suggested for the regulatory role of lncRNAs in physiological functions, including trans- and cis-regulatory mechanisms. In a trans-regulatory mechanism, lncRNAs (such as HOTAIR) could affect the transcription of specific genes through their interaction with chromatin-remodeling complexes and complex recruitment to genomic DNA sequences[18]. Some lncRNAs (such as lincRNA-21) also act as cis-regulators by exerting their function on nearby transcripts[19]. Growing evidence has shown that lncRNAs may act as ceRNAs *via* their miRNA response elements for specific miRNA targets, thus blocking the target binding ability of a single miRNA or multiple miRNAs[20,21]. Several lncRNAs have been suggested to function as ceRNAs, including PTENP1[22], H19[23], and CCAT1[24].

In the present study, we investigated the potential role of lncRNA RP4 as a ceRNA of *SH3GLB1* that competes for miRNA-7-5p binding sites, thereby regulating the expression of *SH3GLB1* mRNA targeted by miRNA-7-5p. The overexpression of lncRNA RP4 inhibited colorectal cancer cell proliferation and tumor growth both *in vitro* and *in vivo*, and increased early apoptosis. These findings suggest that lncRNA RP4 plays a critical role in the modulation of colorectal cancer progression.

To further elucidate the role of lncRNA RP4 in colorectal cancer, we analyzed its regulatory mechanism as a ceRNA by bioinformatics analysis and experimental verification. qRT-PCR analysis showed that lncRNA RP4 overexpression downregulated miR-7-5p expression in colorectal cancer cells, while an inverse correlation was detected between lncRNA RP4 and miR-7-5p expression. Additional functional experiments confirmed that miR-7-5p overexpression promoted cell proliferation, while an inverse correlation was detected between miR-7-5p and *SH3GLB1* expression. Consistent with these findings, miR-7-5p has been found to affect cell proliferation, anchorage-independent growth, migration and invasion, apoptosis, and chemosensitivity by targeting specific oncogenic genes in various types of tumor[25-27].

SH3GLB1, a membrane curvature-inducing protein, interacts with BECN1 though UVRAG and regulates the post-Golgi trafficking of membrane-integrated ATG9A during autophagy[28]. In the present study, we found that lncRNA RP4 overexpression upregulated autophagy. Recently, Takahashi *et al*[29] reported that SH3GLB1 is a haploinsufficient tumor suppressor that functions to prevent the acquisition of apoptosis resistance and malignant transformation during *Myc*-driven lymphomagenesis. Our data supported the tumor suppressor role of SH3GLB1 in colorectal cancer. During tumor development and progression, protein interactions between SH3GLB1 and BAX resulted in the activation of caspase 3, thereby inducing apoptosis[30]. Similarly, we showed that lncRP4-induced *SH3GLB1* upregulation increased levels of BAX and caspase 3 in colorectal cancer cells.

Previous studies observed that dysregulated PI3K/Akt signaling in human colorectal cancer is associated with the growth and proliferation pattern of cancer cells[15,16], while the PI3K/Akt pathway negatively regulates autophagy[31,32]. Consistent with this, we

detected reduced PI3K and Akt phosphorylation in lncRP4-overexpressing colorectal cancer cells.

The present study has a number of limitations. First, because of a lack of colorectal cancer tissue, we could not evaluate the expression pattern of lncRNA RP4, miR-7-5p, or *SH3GLB1* in carcinoma tissues and were thus unable to elucidate the clinical significance of lncRP4 in colorectal cancer. The collection of more colorectal cancer tissue will be necessary to overcome this. Second, we did not use small inhibitors of different signaling pathways, yet it is conceivable that the mechanism of lncRNA RP4 involves multiple modalities.

Taken together, our results demonstrate that lncRNA RP4 plays an important role in the progression of human colorectal cancer by functioning as a ceRNA to regulate the expression of *SH3GLB1* through miR-7-5p sponge activity. The pleiotropic effects of lncRNA RP4 on colorectal cancer pathogenesis suggest that is has the potential to be a therapeutic target for colorectal cancer.

ARTICLE HIGHLIGHTS

Research background

Colorectal cancer is the fourth most common cancer and the fifth most common cause of cancer-related death in China. Surgical resection followed by adjuvant chemotherapy, the most commonly used strategy for colorectal cancer management, has poor treatment response in some patients. Therefore, it is necessary to identify effective therapeutic targets to improve treatment and prognosis.

Research motivation

Long noncoding RNAs (lncRNAs), which may serve as novel therapeutic targets, are involved in the development and progression of human colorectal cancer. In our previous study, lncRNA RP4 was found to be dysregulated in colorectal cancer *via* microarray analysis. This indicated that this lncRNA may play an important role in colorectal cancer. Thus, in the present study, lncRNA RP4 was investigated to find out its role in colorectal cancer progression through an *in vitro* cell model and an *in vivo* xenograft model. Besides, the possible mechanisms in the regulation of lncRNA RP4 had not been well described.

Research objectives

To investigate the role of long noncoding (lnc)RNA RP4 in colorectal cancer, and to find out the possible mechanisms of the regulation.

Research methods

Cell counting kit-8 assay *in vitro* and xenograft tumor model *in vivo* were performed to evaluate the role of lncRNA RP4 in the regulation of proliferation. Annexin V/propidium iodide staining was performed to detect the role of lncRNA RP4 in apoptosis. qPCR and Western blot were performed to identify the relationship between lncRNA RP4 and SH3GLB1. And then, Western blot was done to analyse PI3K/Akt signaling pathway and autophagy pathway in the regulation.

Research results

Both cell counting kit-8 assay *in vitro* and xenograft tumor model *in vivo* showed that lncRNA RP4 could inhibit the proliferation and growth of colorectal cancer cells. lncRNA RP4 could promote early apoptosis. lncRNA RP4 was found to positively regulate SH3GLB1 expression, and correlation analyses further confirmed the existence of a significant correlation between lncRNA RP4 and SH3GLB1 expression. We also observed a positive regulatory effect of miR-7-5p on cell proliferation *via* the negative regulation of SH3GLB1.

Research conclusions

Our results demonstrate that lncRNA RP4 plays an important role in the progression of human colorectal cancer by functioning as a ceRNA to regulate the expression of SH3GLB1 through miR-7-5p sponge activity. The pleiotropic effects of lncRNA RP4 on colorectal cancer pathogenesis suggest that is has the potential to be a therapeutic target for colorectal cancer.

Research perspectives

This study suggests that the lncRNA intervention may be a promising treatment strategy for colorectal cancer. The future study might focus on the specific regulatory role of lncRNA RP4 in colorectal cancer *in vivo*, and the therapeutic effect of lncRNA RP4 needs to be validated in clinical practice.

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