Basic Study

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

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Abstract

AIM
To investigate the role of long noncoding RNA (IncRNA) RP4 in colorectal cancer.
INTRODUCTION

Colorectal cancer is the fourth most common cancer and the fifth most common cause of cancer-related death in China, with an estimated 331 300 newly diagnosed patients and 159 300 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.

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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or IncRNA 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 IncRNA 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) for evaluation of the expression of lncRNA RP4. The expression of β-actin and U6 snRNA genes was assessed simultaneously in all samples as an internal control for IncRNA/mRNA and miRNA expression, respectively. Relative gene expression was determined by the 2^ΔΔCT method. Oligonucleotide primers specific for IncRNA 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 bichinchoninic 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 the primary antibodies, then 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 × 10^5 cells) were seeded in 96-well plates in complete medium and infected with IncRNA RP4, IncRNA 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 IncRNA RP4, IncRNA 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.

**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 IncRNA RP4, SH3GLB1, and miR-7-5p were performed using Pearson’s correlation coefficient. A P-value < 0.05 was considered statistically significant.

| Table 1  Sequences of the primers used |
|-----------------------------|-----------------------------|-----------------------------|
| Gene                        | Forward primer (5'-3')      | Reverse primer (5'-3')      |
| ENST00000565575             | ATCGGTTCCAATACCTGTCTG       | TCCAAAGCGATGCTATGCTG       |
| SH3GLB1                     | CGCTGTCTGAATGACTTTGT        | CCTTCTGCTGCCACTACAC        |
| β-actin                     | GTGGCCGAGACTTTGTG           | CCTGAACAAAGCAGCTCATATT     |

**RESULTS**

**IncRNA RP4 regulates proliferation, tumor growth, and early apoptosis in colorectal cancer cells**
To investigate the role of IncRNA 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 IncRNA RP4, while early apoptosis was positively regulated by IncRNA RP4 (Figure 1C and D). These results suggested that IncRNA RP4 exerts a negative regulatory role in colorectal cancer cell
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 proliferation and a positive regulatory role in early apoptosis of colorectal cancer cells.

**LncRNA RP4 inhibits the growth of colorectal cancer cells by regulating SH3GLB1**

Compared with the control group, colorectal cancer cells 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.

**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. \( P < 0.001 \) for between-group comparisons.
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.

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.
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. *P < 0.05, **P < 0.01, and ***P < 0.001 for between-group comparisons.
SH3GLB1 expression (Figure 2).

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

Because no direct interaction exists between IncRNA RP4 and SH3GLB1, we further analyzed the potential functional mechanism by the introduction of miRNA. IncRNAs 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 IncRNA 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 IncRNA 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 IncRNA-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 IncRNA-RP4 on intracellular signaling revealed that IncRNA-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 IncRNA-RP4 in colorectal cancer cells (Figure 4B). Taken together, we propose a schematic whereby IncRNA 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 IncRNAs 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 IncRNAs in physiological functions, including trans- and cis-regulatory mechanisms. In a trans-regulatory mechanism, IncRNAs (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 IncRNAs (such as lincRNA-21) also act as cis-regulators by exerting their function on nearby transcripts [19]. Growing evidence has shown that IncRNAs 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 IncRNAs 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 IncRNA RP4 as a ceRNA of SH3GLB1 that competes for miRNA-7-5p binding sites, thereby regulating the expression of SH3GLB1 mRNA targeted by miR-7-5p. The overexpression of IncRNA RP4 inhibited colorectal cancer cell proliferation and tumor growth both in vitro and in vivo, and increased early apoptosis. These findings suggest that IncRNA RP4 plays a critical role in the modulation of colorectal cancer progression.

To further elucidate the role of IncRNA RP4 in colorectal cancer, we analyzed its regulatory mechanism as a ceRNA by bioinformatics analysis and experimental verification. qRT-PCR analysis showed that IncRNA RP4 overexpression downregulated miR-7-5p expression in colorectal cancer cells, while an inverse correlation was detected between IncRNA 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 tumors [25-27].

SH3GLB1, a membrane curvature-inducing protein, interacts with BECN1 though UVRA8 and regulates the post-Golgi trafficking of membrane-integrated ATG9A during autophagy [28]. In the present study, we found that IncRNA 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 IncRP4-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 IncRNA RP4 involves multiple modalities.

Taken together, our results demonstrate that IncRNA 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 IncRNA RP4 on colorectal cancer pathogenesis suggest that it has the potential to be a therapeutic target for colorectal cancer.

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, IncRNA RP4 was found to be dysregulated in colorectal cancer via microarray analysis. This indicated that this IncRNA may play an important role in colorectal cancer. Thus, in the present study, IncRNA 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 IncRNA 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 IncRNA RP4 in the regulation of proliferation. Annexin V/propidium iodide staining was performed to detect the role of IncRNA RP4 in apoptosis. qPCR and Western blot were performed to identify the relationship between IncRNA 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 IncRNA RP4 could inhibit the proliferation and growth of colorectal cancer cells. IncRNA RP4 could promote early apoptosis. IncRNA RP4 was found to positively regulate SH3GLB1 expression, and correlation analyses further confirmed the existence of a significant correlation between IncRNA 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 IncRNA 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 IncRNA RP4 on colorectal cancer pathogenesis suggest that it has the potential to be a therapeutic target for colorectal cancer.

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

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