LncRNA RP11-757G1.5 sponges miR-139-5p and upregulates YAP1 thereby promoting the proliferation and liver, spleen metastasis of colorectal cancer

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Abstract

Background

Accumulating evidence indicates that long non-coding RNAs (lncRNAs) play a crucial role in tumorigenesis. However, the biological functions of lncRNAs in colorectal cancer (CRC) remain unclear.

Methods

Microarray dataset analysis, FISH, RT-qPCR was used to detect RP11-757G1.5 expression in CRC tissues. The biological function of RP11-757G1.5 in CRC was determined by colony formation, Edu cell proliferation, wound healing and transwell assays. Bioinformatics binding site analysis, Luciferase reporter assay, Ago2 immunoprecipitation assays and western blot were performed to demonstrate the mechanism of RP11-757G1.5 acts as a molecular sponge of miR-139-5p. Moreover, we further explore the potential role of RP11-757G1.5 in CRC orthotopic xenografts.

Results

We discovered a novel lncRNA RP11-757G1.5, that was overexpressed in CRC tissues, especially in aggressive cases. Moreover, upregulation of RP11-757G1.5 strongly correlated with poor prognosis of patients with CRC. Functional analyses revealed that RP11-757G1.5 promotes cell proliferation in vitro and in vivo. Furthermore, RP11-757G1.5 enhanced cell migration and invasion in vitro and in vivo. Mechanistic studies demonstrated that RP11-757G1.5 regulated the expression of YAP1 through sponging miR-139-5p and inhibiting its activity thereby promoting CRC progression and development.

Conclusions

Altogether, these results reveal a novel RP11-757G1.5/miR-139-5p/YAP1 regulatory axis that participates in CRC progression and development.

Background

Colorectal cancer (CRC) is the third most common cancer worldwide[1]. The occurrence and development of CRC involve a series of complex changes at the genetic and epigenetic levels[2]. CRC is difficult to treat at all stages due to the high rates of cancer metastasis, recurrence and chemoresistance. This highlights the need for the development of improved therapeutic strategies. Numerous studies have demonstrated that long non-coding RNAs (lncRNAs) are involved in the occurrence and development of CRC.
LncRNAs are a class of non-protein coding RNA molecules that consist of > 200 nucleotides. LncRNAs have been reported to regulate a broad range of functions including post-transcriptional and chromatin modification. They are also associated with the development of various cancers including liver[3], colorectal[4], gastric [5]and small cell lung cancer[6]. MicroRNAs (miRNAs) are a class of non-coding RNAs known to modulate various aspects of tumorigenesis and cancer progression[7, 8], including gene expression. Evidence from recent studies show that some LncRNAs modulate gene expression by suppressing miRNA levels. Specifically, LncRNAs bind to miRNA, making them unavailable for interaction with mRNA, thereby acting as competitive endogenous RNA (ceRNA). This sponging of miRNAs lifts the suppression of gene expression by miRNAs.

Numerous studies have reported that miR-139-5p is a tumor suppressor in CRC[9-11]. Decreased levels of miR-139-5p have been linked with enhanced tumor size, lymph node metastasis and advanced tumor grade[12, 13]. Moreover, miR-139-5p inhibits CRC progression by blocking Notch1[9, 14]. However, it has not been established whether LncRNAs interact with miR-139-5p to modulate CRC.

Here, we uncovered RP11-757G1.5, a novel LncRNA that is highly expressed in CRC tissues. Kaplan-Meier analysis, log-rank test, univariate and multivariate analyses indicated that upregulated RP11-757G1.5 correlated with higher proliferation, invasion of CRC cells in vitro and in vivo, as well as poor prognosis of CRC. Our findings show that RP11-757G1.5 directly interacts with miR-139-5p, acting as a miRNA decoy to regulate YAP1 expression. Taken together, our findings highlight the therapeutic potential of RP11-757G1.5 in CRC.

Materials And Methods

Clinical samples

CRC tissues and adjacent non-cancer control tissue were obtained from patients diagnosed with CRC at the Department of general surgery, at the second affiliated hospital of Nanchang University. All patient specimens were collected with patients’ written informed consent. The clinical information of all participants is summarized in Table 1. Ethical approval for this study was provided by the clinical research ethics committee of the second affiliated hospital of Nanchang University.
### Table 1
The correlation of the expression of RP11-757G1.5 with clinical features in Colorectal cancer

| Characteristics          | Number of case | RP11-757G1.5 expression | p value |   |
|--------------------------|----------------|--------------------------|---------|---|
|                          |                | High (n = 56)            | Low (n = 56) |       |
| Gender                   |                |                         |         |   |
| Male                     | 63 (56.3%)     | 30                       | 33      | p = 0.568 |
| Female                   | 49 (43.7%)     | 26                       | 23      |   |
| Age at diagnosis         |                |                         |         |   |
| < 60                     | 32 (28.6%)     | 15                       | 17      | p = 0.676 |
| ≥ 60                     | 80 (71.4%)     | 41                       | 39      |   |
| Differentiation          |                |                         |         |   |
| Poor                     | 48 (42.9%)     | 23                       | 25      | p = 0.404 |
| Moderately               | 28 (25.0%)     | 17                       | 11      |   |
| Well                     | 36 (32.1%)     | 16                       | 20      |   |
| Tumor size (cm)          |                |                         |         |   |
| < 5                      | 54 (48.2%)     | 19                       | 35      | p = 0.003** |
| ≥ 5                      | 58 (51.8%)     | 37                       | 21      |   |
| Depth of invasion        |                |                         |         |   |
| T1,T2                    | 40 (35.7%)     | 19                       | 21      | p = 0.693 |
| T3,T4                    | 72 (64.3%)     | 37                       | 35      |   |
| Location                 |                |                         |         |   |
| Transverse colon         | 30 (26.8%)     | 13                       | 17      | p = 0.779 |
| Ascending colon          | 34 (30.4%)     | 19                       | 15      |   |
| Descending colon         | 19 (17.0%)     | 9                        | 10      |   |
| Sigmoid colon            | 29 (25.8%)     | 15                       | 14      |   |
| Lymph node status        |                |                         |         |   |
| N0                       | 52 (46.4%)     | 33                       | 19      | p = 0.008** |
| N1 + N2                  | 60 (53.6%)     | 23                       | 37      |   |
| TNM stage                |                |                         |         | p < 0.001*** |
| I + II                   | 61 (54.5%)     | 20                       | 41      |   |
| III + IV                 | 51 (45.5%)     | 36                       | 15      |   |

**p < 0.01; ***p < 0.001

### Reagents

Antibodies against YAP1 (Cat. No. 14074T), Cyclin D1 (Cat. No. 2922S) and PCNA (Cat. No. 13110T) were purchased from Cell signaling technology. Antibodies against GAPDH (Cat. No. ab8245) and Tubulin (Cat. No. ab210797) were purchased from Abcam. MiR-139-5p mimic, miR-139-5p inhibitor and YAP1 siRNA (si-YAP1) were purchased from GenePharma (Suzhou, China).

### Cell culture

The CRC cell lines, HT-29, HCT-116, SW480, SW620, LoVo and Caco-2, as well as the normal human colonic epithelial cell line NCM460 were purchased from American type culture collection (ATCC, Manassas, VA). All cells were cultured in DMEM (Invitrogen, Grand Island, NY, USA) supplemented with 10% FBS (Gibco, Cat. No. 10100147), 1% L-glutamine (Thermo Fisher Scientific, Cat. No. 21051024), 25 units/ml penicillin (Gibco, Cat. No. 15140148) and 25 g/ml streptomycin (Gibco, Cat. No. 15140148) Cell lines were authenticated and were regularly tested for mycoplasma as per ATCC’s guidelines.

### RNA extraction and RT-qPCR assay
RNA extraction was done using Trizol (Invitrogen, Cat. No. **15596-026**). Reverse transcription was done using Superscript III transcriptase kit (Invitrogen, Cat. No. 18080-044). RT-qPCR was conducted on the Biorad CFX96 system using SYBR green. Primer sequences used are shown in Table 4. RT-qPCR was done using the following protocol: 55°C for 3 minutes, 95°C for 7.5 minutes, followed by 50 cycles at 95°C for 10 seconds, and 65°C for 2 minutes. The extension step was done at 95°C for 2 minutes, 50°C for 1 minute, and 50°C for 10 seconds. GAPDH was used as the reference gene. The PureLink® miRNA kit was used for extraction of miRNAs. The RT-qPCR protocol was as follows: 95°C for 3 minutes, followed by 50 cycles at 95°C for 10 seconds, and 55°C for 50 seconds. The reference genes were U6 and/or β-actin.

Table 4 The sequences for RT-qPCR

|          | F                                         | R                                         |
|----------|-------------------------------------------|-------------------------------------------|
| GAPDH    | 5′-TGTGGGCATCAATGGATTTGG-3′                | 5′-ACACCATGTATTCCGGGTTCAAT-3′             |
| RP11-757G1.5 | 5′-CGTAGGAAAAAGCGGTACCGAT-3′            | 5′-AAAAGCGGAAGCAGCAAAGT-3′              |
| miR-139-5p | 5′-ACACTCCAGCTGCACGTGTC-3′                | 5′-TGGTGTCGTGGAGTCGGTTGA-3′              |
| YAP1     | 5′-CGCTGACGGAGTACAAGTG-3′                 | 5′-GTAGGAGCCGACCTCGTTG-3′               |

Isolation of cytoplasmic and nuclear RNA

Cytoplasmic and nuclear RNA isolation and purification were done using cytoplasmic & nuclear RNA purification kit (Norgen, Cat. No. 210000) following manufacturer’s instructions.

Plasmid construction and transfection
Sh-RP11-757G1.5#1 (sh-757G1.5#1), sh-RP11-757G1.5#2 (sh-757G1.5#2) and pcDNA3.1-RP11-757G1.5 (pcDNA3.1-757G1.5) along with the respective controls (sh-NC and vector) were purchased from GenePharma (Suzhou, China). shRNA sequences are shown in Table 3. 1×10^6 CRC cells/well were seeded into 6-well plates and cultured until they were 50%-60% confluent. They were then transfected with plasmid using lipofectamine 3000 (Thermo Fisher Scientific, Cat. No. L3000008) following the manufacturer's instructions. Viral particles were collected by centrifuging at 1000 rpm for 5 minutes at 4°C. These virus particles were centrifuged and filtered used to infect HCT-116 and SW480 cells to generate stable overexpression and knockdown cells, respectively.

Table 3 The sequences of shRNA for RP11-757G1.5

| sh-NC: 5'-GCAGATGCCCCATTGGCCA-3' |
| sh-RP11-757G1.5#1: 5'-ATCCGGCGGTAGCTAGCTAAGCAA-3' |
| sh-RP11-757G1.5#2: 5'-GAAAATTGCCTACAGGAGGTCA-3' |
| pcDNA3.1-RP11-757G1.5 |
| F: 5'-AGGGCCTCCGCTGAATTTTAAAGGTCCCTGGAAG-3' (BamHI) |
| R: 5'-CAAGTCGAGGGTGCGTACCCGATAC-3' (XhoI) |
| miRNA-139-5p mimic: 5'-ACCGGTGAATAGTGCGATGCTACCCGGTAAGGCGCCG-3' |
| miRNA-139-5p inhibitor: |
| 5'-AAGCTGAGGGTGCGTACCCGTCATTAA-3' |
| si-NC: 5'-AATTGCGGAACCGGTTGGATTAA-3' |
| si-YAP1: 5'-GAAGGCAGTGAAGTGAGCTA-3' |

**Cell proliferation assay**

CRC proliferation was assessed by colony formation and Edu incorporation assays. For colony formation analysis, 350 transfected cells/well were seeded into 6-well plates and cultured for 2 weeks. Colonies were then fixed with 4% paraformaldehyde before staining with 0.5% crystal violet and the number of colonies was counted. Edu assays were done using a commercial kit (Ribobio, Cat. No.)
C10310) according to the manufacturer’s instructions. All assays were done in triplicate.

**Cell invasion assay**

Cell migration was examined using wound-healing assays. When cells were about 100% confluent, media was removed and a 10μl tip used to scrape the monolayer vertically. Cells were then washed 3 times with PBS 1X to remove cell fragments and then put back in culture. The cells were imaged at 0 and 48 hours after wounding using an inverted microscope (Olympus). Wound healing capacity was estimated based on the size of the gaps measured under the microscope.

For cell migration assays, CRC cells/well were seeded into 24-well plates after treatment as indicated and then cultured for 72 hours. The upper chambers of transwell plates were coated with Corning Matrigel (BD Biocoat, Cat. No. 354234) for 2 hours prior to seeding. The cells were resuspended in serum-free media at 1×10^5 cells/ml and seeded into the upper chambers. 700μl of cell culture media supplemented with 10% FCS was then added into the lower chambers and the cells incubated at 37°C in normal cell culture conditions for 10-15 hours. Cells that invaded the lower chamber were fixed with by methanol for 15 minutes at room temperature and stained with 0.1% (w/v) crystal violet in the dark.

**Luciferase reporter assay**

HCT-116 cells were cotransfected with pLuc, pRL-CMV, miR-139-5p mimic (negative control, NC), and pcDNA3.1-RP11-757G1.5 (pcDNA3.1) and then subjected to luciferase assays using the dual-luciferase® reporter system (Beyotime, Cat. No. E1960).

**RNA pull-down assay**

HCT-116 cells were lysed in 1 ml of cell lysis buffer for 72 hours. 1.5μL of RNase inhibitor, 10μL of streptavidin agarose beads and 500pM of antisense oligos were added and the cells rotated overnight at 4°C. The beads were washed 5 times using cell lysis buffer. The RNA was then analyzed by RT-qPCR analysis.

**Ago2 immunoprecipitation assay**

Transfected cells were lysed with RIPA lysis buffer and centrifuged for 20 minutes at 12000 rpm. 2μl of AGO2 antibody and 10μl of beads were added and the supernatant rotated overnight at 4°C. The
mixture was washed 3 times with lysis buffer and RNA using Trizol reagent (Invitrogen, Cat. No. 15596-026).

**Western blotting**

Proteins were separated by electrophoresis on 8% or 10% SDS-PAGE gels and then transferred onto 0.45μm PVDF membranes. The membranes were then blocked with non-fat milk for xx at xx. Both antibodies were diluted in xx at 1:1000.

**Immunohistochemistry analysis**

IHC analysis of YAP1 was done using the Dako Envision™ FLEX + system (Dako, Glostrup, Denmark) as described previously[15].

**In vivo studies**

Twenty-four 6-8-week-old nude mice were purchased from the Shanghai laboratory animal company. HCT-116 and SW480 cells expressing a luciferase reporter (pcDNA3.1-luciferase) and stably expressing pcDNA3.1-757G1.5 and sh-757G1.5#1, were generated. Next, 1×10^6 HCT-116 and SW480 cells (mixed with Matrigel at a 1:1 ratio), were injected into the subrenal capsule. Tumor formation and metastasis were monitored weekly using an IVIS fluorescent imaging system (IVIS Spectrum). The mice were sacrificed after 6 weeks and tumors harvested for analysis.

**Statistical analyses**

Data are presented as the mean ± S.D. Student’s t-test, the Mann-Whitney U-test and the χ2 test were used to analyze differences between groups. Survival rates were evaluated using Kaplan-Meier analysis and compared by the log-rank test. HRs and 95% CIs were calculated using Cox proportional hazards model. P-value < 0.05 was considered statistically significant.

**Results**

**Overexpression of RP11-757G1.5 in CRC associates with poor prognosis**

To identify IncRNAs that are differentially expressed in CRC, we analyzed microarray dataset GSE63675 from GEO, which consists of IncRNAs data for 43 CRC tissues and 6 adjacent non-tumor control tissue. Notably, 8 IncRNAs were differentially expressed in CRC tissues relative to adjacent non-tumor tissues (Fig. 1A). Among
them, IncRNA RP11-757G1.5 was selected for further analysis. Next, we evaluated the expression of RP11-757G1.5 in CRC tissues by RT-qPCR and observed that this IncRNA was significantly upregulated in CRC tissues relative to the non-tumor control tissue \((p < 0.001, \text{Fig. 1B})\). Subsequently, we evaluated the relationship between high RP11-757G1.5 expression and clinicopathological features of the disease. Results indicated that elevated RP11-757G1.5 correlated with greater lymph node metastasis and advanced TNM staging (Fig. 1C-D). To assess the significance of this association, we divided 112 CRC patients into 2 groups depending on the level of RP11-757G1.5 expression: RP11-757G1.5-high and RP11-757G1.5-low. Pearson chi-square analysis or Fisher's Exact tests revealed that elevated RP11-757G1.5 levels correlated with larger tumor size \((p = 0.003)\), lymph node metastasis \((p = 0.008)\) and advanced TNM staging \((p < 0.001)\). No apparent association was observed between RP11-757G1.5 levels and other clinical features (Table 1). Next, Kaplan-Meier analysis and log-rank test were done to establish the relationship between RP11-757G1.5 and CRC survival time. This analysis showed that patients in the RP11-757G1.5-high group exhibited a significantly shorter survival rate relative to those in the RP11-757G1.5-low group \((45.741 ± 3.539 \text{ vs } 69.818 ± 3.662 \text{ months}; \text{log rank } = 4.178, \ p = 0.0047, \text{Fig. 1E})\). Moreover, high RP11-757G1.5 levels correlated with poor disease-free survival \((\text{log rank } = 9.561, \ p = 0.0129, \text{Fig. 1F})\). Univariate and multivariate analyses revealed RP11-757G1.5 expression as an independent prognostic indicator of CRC (hazard ratio \((\text{HR}) = 3.441, 95\% \text{ confidence interval } (\text{CI}) = 1.471–8.005, \ p = 0.008; \text{HR} = 2.015, 95\% \text{ CI } = 1.018–5.856, \ p = 0.019, \text{Table 2})\). Taken together, these findings show that high RP11-757G1.5 levels correlate with poor CRC clinical outcomes.

Table 2

| Variable                | Subset                  | Univariate analysis | Multivariate analysis |
|-------------------------|-------------------------|---------------------|-----------------------|
|                         |                         | p-value             | HR (95\% CI)          | p-value | HR (95\% CI)          |
| Gender                  | Male/female             | 0.771               | 0.734 (0.415–1.859)   | --      | --                    |
| Age at diagnosis(years) | < 60/≥60                | 0.474               | 0.626 (0.337–2.552)   | --      | --                    |
| Differentiation         | Well + moderately/poorly| 0.331               | 1.422 (0.614–2.683)   | --      | --                    |
| Tumor size (cm)         | < 5/≥5                  | 0.002**             | 2.742 (0.326–4.663)   | 0.017*  | 3.471 (0.527–6.028)   |
| Depth of invasion       | T1 + T2/T3 + T4         | 0.095               | 1.116 (0.549–2.421)   | --      | --                    |
| Location                | Colon/rectum            | 0.852               | 0.632 (0.412–1.743)   | --      | --                    |
| Lymph node status       | N0/N1 + N2              | 0.153               | 2.571 (1.306–4.663)   | 0.216   | 2.154 (1.146–6.915)   |
| TNM stage               | I + II/III + IV         | 0.003**             | 13.145 (5.044–34.378) | 0.004** | 7.603 (2.521–33.109)  |
| RP11-757G1.5            | High/low                | 0.008**             | 3.441 (1.471–8.005)   | 0.019*  | 2.015 (1.018–5.856)   |

RP11-757G1.5 promotes CRC cell proliferation in vitro

Next, we evaluated the expression of RP11-757G1.5 in normal NCM460 and CRC cell lines (HT-29, HCT-116,
SW480, SW620, LoVo, Caco-2) by RT-qPCR. The results revealed significantly higher levels of RP11-757G1.5 in CRC cell lines relative to NCM460 ($p < 0.05$, Fig. 2A). Among the CRC cell lines, RP11-757G1.5 expression was highest in SW480 ($p < 0.001$) and least in HCT-116 ($p < 0.05$). These 2 CRC cell lines were therefore selected for downstream experiments. In addition, fluorescence in situ hybridization (FISH) results suggested that IncRNA RP11-757G1.5 mainly located in the cytoplasm (Fig. 2B). To investigate the biological function of RP11-757G1.5 in CRC, RP11-757G1.5 was overexpressed in HCT-116 cells (Fig. 2C) and knocked down in SW480 cells (Fig. 2D). To minimize the chances of off-targeting, 3 shRNAs (sh-RP11-757G1.5#1 (or sh-757G1.5#1) and sh-RP11-757G1.5#2 (or sh-757G1.5#2)) were used (Fig. 2D). RT-qPCR was conducted to confirm the success of RP11-757G1.5 overexpression and knockdown, and results are shown in Fig. 2E-F, ($p < 0.05$). Further analyses showed that RP11-757G1.5 overexpression significantly enhanced HCT-116 proliferation and colony formation while these processes were suppressed by RP11-757G1.5 silencing in SW480 cells (Fig. 3A-D). Western blot analysis showed that overexpression of RP11-757G1.5 in HCT-116 enhanced Cyclin D1 and PCNA expression, factors known to promote cell proliferation (Fig. 3E) while RP11-757G1.5 knockdown diminished their expression (Fig. 3F). Taken together, these datasets suggest that RP11-757G1.5 may promote CRC progression.

RP11-757G1.5 promotes CRC cell migration and invasion in vitro

Next, we performed cell invasion and migration assays to determine the role of RP11-757G1.5 in CRC metastasis. We found that RP11-757G1.5 overexpression promoted HCT-116 cell migration and invasion (Fig. 4A-C). Conversely, RP11-757G1.5 knockdown suppressed these processes in SW480 cells (Fig. 4B-D). Taken together, these results suggest that the RP11-757G1.5 promotes CRC cell migration and invasion in vitro.

LncRNA RP11-757G1.5 acts as a molecular sponge of miR-139-5p to regulate YAP1 expression in CRC cells

To understand the mechanisms by which RP11-757G1.5 promotes CRC, we evaluated its subcellular localization in HCT-116 and observed that it predominantly resided in the cytosol (Fig. 5A). This suggests it may function at the post-transcriptional level. Multiple studies have reported that IncRNAs may act as a sponge by binding competitively to miRNA, thereby modulating gene expression by making the unavailable for interaction with their target miRNA responsive elements (MREs). To investigate whether RP11-757G1.5 performs this role, we examined possible binding sites between miR-139-5p and RP11-757G1.5 by cloning the full-length RP11-757G1.5 containing the presumptive miR-139-5p binding sites (Fig. 5B). This analysis was done on the background that...
miR-139-5p and RP11-757G1.5, exhibit opposing functions in CRC. We therefore constructed a luciferase reporter plasmid (pLuc) containing RP11-757G1.5 (pLuc-RP11-757G1.5-WT) or a mutant bearing mutation in the miR-139-5p seed sequence (pLuc-RP11-757G1.5-Mut). Next, we examined the association between miR-139-5p and RP11-757G1.5 by luciferase reporter assays and observed that overexpressing miR-139-5p (or its miR-139-5p mimic) markedly suppressed pLuc-RP11-757G1.5-WT activity relative to the mutant reporter (Fig. 5C), confirming that miR-139-5p binds and inhibits RP11-757G1.5. To test whether this interaction is physical, we carried out RNA immunoprecipitation (RIP) with an anti-Ago2 antibody. We found enrichment for both RP11-757G1.5 and miR-139-5p in the Ago2 complex (Fig. 5E).

**RP11-757G1.5 modulates YAP1 expression by competitively binding miR-139-5p**

Several lines of evidence have shown that microRNAs modulate CRC pathogenesis via YAP1 regulation[16-18]. Using bioinformatics analysis, we found that miRNAs sequences are compatible with recognition sequences on RP11-757G1.5 and the 3'-UTR of YAP1, suggesting that miR-139-5p may interact with the RP11-757G1.5 sequence and its 3'-UTR (Figure 5B). Next, we tested whether the effects of RP11-757G1.5 on CRC pathogenesis are mediated by the miR-139-5p/YAP1 pathway. Thus, we evaluated the interaction between RP11-757G1.5, miR-139-5p, and YAP1 by luciferase assays and observed that, relative to the empty vector control, RP11-757G1.5 overexpression reversed the miR-139-5p-mediated inhibition of pLuc-NOTCH1-3'UTR luciferase output (Figure 5D), indicating that RP11-757G1.5 suppresses miR-139-5p-mediated inhibition of YAP1 by competitively binding miR-139-5p. Additionally, we found that YAP1 was elevated in CRC tissues and cell lines (Figure 5G-H). RP11-757G1.5 knockdown remarkably suppressed endogenous YAP1 levels in CRC cells (Figure 5F-L). Conversely, YAP1 expression was elevated by RP11-757G1.5 overexpression in CRC cells (Figure 5F-L). Next, we examined the relationship between RP11-757G1.5, miR-139-5p, and YAP1 expression in the GSE63675 dataset. The results showed that RP11-757G1.5 expression negatively correlated with miR-139-5p levels ($r=-0.402$, $p=0.003$), but positively correlated with YAP1 levels ($r=0.380$, $p=0.006$). Additionally, a negative correlation was observed between miR-139-5p and YAP1 expression in the GSE63675 dataset ($r=-0.297$, $p=0.004$) (Figure 5K). To investigate whether the effects of RP11-757G1.5 on YAP1 levels depend on miR-139-5p, HCT-116 cells were co-transfected with the miR-139-5p mimic and pcDNA3.1-RP11-757G1.5 (or pcDNA3.1-757G1.5) and the effects of this approach on YAP1 were evaluated. Results revealed a significantly higher protein levels of YAP1 in HCT-116
cells relative to cells transfected with miR-139-5p mimic. RP11-757G1.5 knockdown markedly reversed the inhibitory effects of miR-139-5p on YAP1 expression in SW480 cells, as revealed by western blot analysis (Figure 5M).

**RP11-757G1.5 promotes tumor progression in CRC via miR-139-5p/YAP1 axis**

MiR-139-5p and YAP1 have been reported to modulate CRC progression [9, 19-21]. We therefore wondered whether RP11-757G1.5 regulates the pathogenesis of CRC via miR-139-5p/YAP1 axis. To investigate this, we evaluated how miR-139-5p and YAP1 affect RP11-757G1.5-driven cell proliferation. This analysis revealed that miR-139-5p overexpression or YAP1 knockdown blocked RP11-757G1.5-driven CRC cell proliferation (Figure 6A-D). Next, western blotting was performed to investigate whether miR-139-5p and YAP1 affect CyclinD1 and PCNA levels in the context of RP11-757G1.5-driven cell proliferation. Results showed that miR-139-5p expression or YAP1 knockdown significantly reversed the effects of RP11-757G1.5 overexpression on CyclinD1 and PCNA expression (Figure 6E-F). Next, we assessed how the miR-139-5p/YAP1 axis affects RP11-757G1.5 driven migration and invasion of CRC cells. The results revealed that miR-139-5p expression or YAP1 silencing suppressed cell migration and invasion caused by RP11-757G1.5 overexpression in CRC cells (Figure 6G-J).

Collectively, these data demonstrate that RP11-757G1.5 acts as an oncogene in CRC, at least in part by sponging miR-139-5p, thereby modulating YAP1 levels.

**Deregulation of IncRNA RP11-757G1.5 suppresses cell proliferation and invasion in CRC orthotopic xenografts**

Next, we investigated the role of shRP11-757G1.5 in CRC in vivo. Briefly, HCT-116 cells overexpressing RP11-757G1.5 or with SW480 knockdown were xenografted into mice subcutaneously. The effect of RP11-757G1.5 knockdown on tumor growth, tumor growth and metastasis was monitored using IVIS imaging system. This analysis revealed that tumor luciferase activity in the pcDNA3.1-757G1.5 expressing cells was higher than in those transfected with an empty vector (Figure 7A). Additionally, we found that RP11-757G1.5 overexpression enhanced tumor growth and metastasis (Figure 7A-E). RP11-757G1.5 knockdown suppressed tumor growth and metastasis (Figure 7F-J). Additionally, overexpression of RP11-757G1.5 promoted metastasis to the liver and spleen (Figure 7K-L). Taken together, these observations indicate that RP11-757G1.5 is an oncogene in CRC.

**Discussion**
Accumulating evidence has underscored the significance of lncRNAs in the tumorigenesis and progression of CRC. Besides the widely studied lncRNAs, HOTAIR[22], MALAT1[23] and H19[24], other lncRNAs participate in CRC pathogenesis. To identify such lncRNAs, we analyzed publicly available CRC microarray data. We uncovered a novel lncRNA RP11-757G1.5, which was highly expressed in CRC tissues and cell lines relative to the adjacent non-cancer tissues. Our data indicate that high RP11-757G1.5 expression positively correlates with larger tumor size, lymph node metastases, and high TNM staging as well as CRC poor clinical outcomes. LncRNAs modulate a wide range of biological processes, including tumor development and progression. This RNA class, therefore, has the potential for application in the diagnosis, treatment, and prediction of CRC clinical outcomes.

Loss-of-function assays showed that silencing of RP11-757G1.5 significantly suppressed CRC proliferation, invasion, and migration in vitro and in vivo, suggesting an oncogenic role for RP11-757G1.5 in CRC. LncRNAs have been suggested to exert their biological functions by acting as ceRNAs, which by sponging miRNAs render them unavailable for interaction with target mRNA. The lncRNA DANCR, has been reported to act as a ceRNA for miR-335-5p and miR-1972, thereby enhancing ROCK1-mediated osteosarcoma pathogenesis[25]. The lncRNA MCM3AP-AS1 enhanced hepatocellular carcinoma by targeting the miR-194-5p/FOXA1 axis[26]. Elsewhere, LINC00152 was reported to drive CRC proliferation, metastasis, and 5-Fu resistance by inhibiting miR-139-5p[9].

Another study found that miR-139-5p was significantly downregulated in CRC tissues which inhibited CRC development, proliferation and metastasis but promoted apoptosis and cell cycle arrest by targeting Notch1 signaling[14]. A variety of microRNAs have been reported to promote CRC carcinogenesis by modulating YAP1 signaling[27-29]. We therefore hypothesized that lncRNA RP11-757G1.5 might modulate CRC progression by targeting miR-139-5p-YAP1 activity. To test this possibility, we used luciferase reporter assays to assess the interaction between possible MREs and lncRNA RP11-757G1.5. As expected, we observed that miR-139-5p repressed the binding of lncRNA RP11-757G1.5 to its targets.

Analysis of the subcellular localization of RP11-757G1.5 in CRC cells revealed that it resides in the cytosol at a similar location to that of miR-139-5p. Additionally, RP11-757G1.5 silencing markedly enhanced miR-139-5p levels in CRC cells. Further analysis revealed that RP11-757G1.5 levels inversely correlated with miR-139-5p levels. Subsequent luciferase reporter assays and RNA pull-down revealed that RP11-757G1.5 physically
interacted with miR-139-5p, thereby sponging miR-139-5p.

YAP1 has been implicated in various cellular processes and cancers[30, 31, 32]. However, the mechanism through which IncRNA regulates the function of YAP1 remains unclear. Here, we identified YAP1 as a direct target of miR-139-5p in CRC. Notably, we find that RP11-757G1.5 and miR-139-5p exhibit opposing functions in CRC, with RP11-757G1.5 promoting cancer progression and miR-139-5p suppressing it. Additionally, in CRC, a positive correlation was observed between RP11-757G1.5 and YAP1, while an inverse relationship was found between miR-139-5p and YAP1. Restoration of YAP1 suppressed the proliferation, migration and invasion of cells induced by RP11-757G1.5 knockdown.

Conclusion

Here, we uncovered a novel lncRNA that is significantly upregulated in CRC. Our data indicate that RP11-757G1.5 correlates with poor CRC prognosis and its silencing suppresses CRC cell proliferation, migration, and invasion in vitro and in vivo. Mechanistically, we show that RP11-757G1.5 exerts its oncogenic function by sponging miR-139-5p, thereby upregulating YAP1 expression. Collectively, our findings highlight the potential of RP11-757G1.5 as a biomarker and therapeutic target in CRC.

Declarations

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Author's contribution

Project design and conception: ZZM, ZXJ.

Performed the research and data collection: LC, ZXJ, LQL, BFQ, HC, LK, ZJF, ZWJ, ZZ, LHL, HJ, HCG and ZJF.

Data statistical and analysis: ZXJ, LC, and BFQ.

Drafted the manuscript: ZXJ and LC.

All authors read and approved the final version of the manuscript.

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**Data availability statement**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Ethics approval and consent to participate**

All Procedures performed in studies involving human participants or animals were reviewed and granted by the Ethical Committee of The Second Affiliated Hospital of Nanchang University, China. Informed consent was obtained from all the patients.

**Consent for publication**

Not applicable.

**Competing interests**

The all authors declare that they have no competing interests.

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Figures
LncRNA RP11-757G1.5 expression is upregulated in CRC tissues and is associated with poor prognosis.
A Heat-maps of lncRNAs that were differentially expressed between CRC tissues and matched adjacent normal samples. LncRNA RP11-757G1.5 was the most appropriate lncRNA to select in eight lncRNAs.

The color scale shown below illustrates the relative RNA expression levels; red represents high expression, and blue represents low expression. B Comparison of lncRNA RP11-757G1.5 in CRC tissues (n=64) and normal tissues (n=56) by RT-qPCR. C-D LncRNA RP11-757G1.5 expression at different lymph node metastasis, TNM stages of CRC patients. E-F Kaplan-Meier survival analysis of the overall survival and disease-free survival in two groups defined by low and high expression of RP11-757G1.5 in patients with CRC.
Figure 2

Overexpression and stable knockdown of RP11-757G1.5 in HCT-116 and SW480 cells. A Relative expression of RP11-757G1.5 in six CRC cell lines and a colonic epithelial cell. B Fluorescence in situ hybridization (FISH) assay was conducted to determine the subcellular localization of lncRNA RP11-757G1.5 in HCT-116 and SW480 cells. Nuclei are stained blue (DAPI), and lncRNA RP11-757G1.5 is stained green. Scale bars represent 25 μm. C-D Representative images of HCT-116 and SW480 cells transfected with pcDNA3.1-757G1.5 and sh-757G1.5#1; sh-757G1.5#2, respectively. E-F The validation of overexpression and knockdown efficacy of RP11-757G1.5 in CRC cell lines by RT-qPCR. *p<0.05; **p<0.01.
RP11-757G1.5 promotes CRC cell proliferation in vitro. A-B Effects of RP11-757G1.5 overexpression and knockdown on colony formation in CRC cells. C-D Effects of RP11-757G1.5 overexpression and downregulation on CRC cell proliferation were measured by a Edu assay. E-F Western blot to detect proliferation-associated antigen Cyclin D1 and PCNA expression in HCT-116, SW480 cells transfected with pcDNA3.1-757G1.5 or sh-757G1.5, respectively. *p<0.05; **p<0.01.
RP11-757G1.5 accelerates CRC cell migration and invasion in vitro. A-B Wound-healing assay was used to investigate the horizontal migration ability with RP11-757G1.5 overexpression or knockdown in CRC cells, and relative gap distance was calculated and plotted on a histogram. C-D Migration and invasion assays were used to investigate the vertical migration and invasion abilities with RP11-757G1.5 overexpression or knockdown in CRC cells, and the number of cells was calculated and plotted on a histogram. *p<0.05, **p<0.01, ***p<0.001.
Figure 5

RP11-757G1.5 sponges miR-139-5p and modulate YAP1 expression. A Subcellular localization of RP11-757G1.5 was determined by RT-qPCR in HCT-116 cell line. B miR-139-5p-binding sequence in RP11-757G1.5 and YAP1 3’UTR. A mutation was generated in RP11-757G1.5 in the complementary site for miR-139-5p binding. C Luciferase activity of a luciferase reporter plasmid (pLuc) containing wild-type or mutant RP11-757G1.5 co-transfected with miR-139-5p was determined using the dual luciferase assay. D MiR-139-5p and pLuc plasmid containing YAP1 3’UTRs were co-transfected with pcDNA3.1-757G1.5 or empty vector into HCT-116 cells to verify whether RP11-757G1.5 can function as a ceRNA of miR-139-5p. E Cellular lysates from HCT-116 cells were used for RIP with an anti-Ago2 antibody or IgG antibody. The levels of RP11-757G1.5 and miR-139-5p were detected by RT-qPCR. F and L The expression levels of YAP1 in HCT-116 cells transfected with pcDNA3.1-757G1.5 and SW480 cells transfected with sh-757G1.5 were analyzed by RT-qPCR and western blot. G The elevated expression of YAP1 in tissue level was detected by IHC test, normalized to para-tumor tissue group. H YAP1 was up-regulated in CRC tissue and cell lines as determined by a RT-qPCR, normalized to para-tumor tissue group and NCM460 group, respectively. I-K Correlation between RP11-757G1.5, miR-139-5p, and YAP1 expression in CRC and normal colon specimens as detected by RT-qPCR (n=41). M Western blot assays were performed to
test YAP1 expression after HCT-116 cells were transfected with miR-139-5p mimic or co-transfected with miR-139-5p mimic and pcDNA3.1-757G1.5. Meanwhile, SW480 cells were transfected with miR-139-5p inhibitor or co-transfected with miR-139-5p inhibitor and sh-757G1.5#1. Data from Western Blot assay has been represented as a quantification graph normalized to the levels of GAPDH together with the statistical tests. *p<0.05, **p<0.01, ***p<0.001.

Figure 6

RP11-757G1.5 exerts tumor-promoting function in CRC by regulating the miR-139-5p/YAP1 axis. A-D The increased cell proliferation ability in pcDNA3.1-757G1.5 transfected CRC cells was abolished by ectopic miR-139-5p expression or YAP1 knockdown. The cell proliferation ability was measured by a clone formation and Edu proliferation assays. E Western blotting analysis of proliferation-associated antigens in HCT116 cells transfected with miR-139-5p mimic or co-transfected with miR-139-5p mimic and pcDNA3.1-757G1.5. F Western blotting analysis of the proliferation-associated antigens in HCT-116 cells transfected with si-YAP1 (siRNA) or co-transfected with si-YAP1 and pcDNA3.1-757G1.5. Tubulin acted as a loading control. G-J The increased cell migration and invasion ability in pcDNA3.1-757G1.5 transfected CRC cells were abolished by ectopic miR-139-5p expression or YAP1 knockdown. Cell migration and invasion were measured by wound healing and Transwell assays. *p<0.05, **p<0.01; ***p<0.001.
Deregulation of IncRNA RP11-757G1.5 suppresses CRC cell proliferation and invasion in CRC orthotopic xenografts. A-E Representative IVIS images of tumor size (A), macroscopic appearance (B), tumor growth curves (C), tumor weight (D), and metastasis (E) in pcDNA3.1-757G1.5 group vs control group. F-J Representative IVIS images of tumor size (F), macroscopic appearance (G), tumor growth curves (H), tumor weight (I), and metastasis (J) in sh-757G1.5#1 group vs control group. K-L Representative macroscopic appearance and IVIS image of metastatic foci (white arrows) in liver (K) and spleen (L). Each sample was run in triplicate and in multiple experiments for mean ± SEM. *p<0.05, **p<0.01.