Upregulated LINC01088 Facilitates Malignant Phenotypes and Immune Escape of Colorectal Cancer by Regulating microRNAs/G3BP1/PD-L1 axis

Shukui Wang (sk_wang@njmu.edu.cn)  
Southeast University Medical College  
https://orcid.org/0000-0001-6972-2587

Chenmeng Li  
Southeast University Medical College

Bei Pan  
Southeast University Medical College

Xuhong Wang  
Southeast University Medical College

Jian Qin  
Southeast University Medical College

Xiangxiang Liu  
Southeast University Medical College

Tianyi Gao  
Nanjing Medical University affiliated Nanjing Hospital: Nanjing First Hospital

Huiling Sun  
Nanjing Medical University affiliated Nanjing Hospital: Nanjing First Hospital

Yuqin Pan  
Nanjing Medical University affiliated Nanjing Hospital: Nanjing First Hospital

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Abstract

**Purpose:** Long intergenic non-coding RNA LINC01088 is a newly discovered long non-coding RNA. Its biological function in colorectal cancer (CRC) remains unknown.

**Methods:** Here, 36 paired CRC and para-cancerous tissues were collected. *In vitro*, fluorescence in situ hybridization assay, qPCR, western blotting analysis and cellular functional experiments, RNA immunoprecipitation (RIP) assay and dual-luciferase reporter system analysis were performed. *In vivo*, xenograft tumor mouse models were generated. Besides, patient-derived intestinal organoid (PDO) was used *ex vivo*.

**Results:** We found that LINC01088 was significantly upregulated in colorectal cancer tissues and CRC cell lines compared to adjacent normal tissues and colonic epithelial cells. High LINC01088 expression was associated with poor outcome in CRC. LINC01088 was mainly located in the cytoplasm. LINC01088 knockdown suppressed the proliferation, migration, invasion, and immune escape of colorectal cancer cells. Mechanistically, LINC01088 bound directly to miR-548b-5p and miR-548c-5p that were significantly upregulated G3BP1 expression, altering CRC cell phenotypes. In mouse xenograft models, LINC01088 knockdown restrained tumor growth and lung metastasis. Further, G3BP1 overexpression reversed LINC01088-knockdown mediated inhibitory effects on tumor growth. Notably, LINC01088 knockdown downregulated PD-L1 expression, while G3BP1 overexpression restored PD-L1 expression in xenograft tumors. Besides, LINC01088 knockdown repressed CRC organoid growth *ex vivo*.

**Conclusion:** Overall, these findings suggested that LINC01088 directly targeted miR-548b-5p and miR-548c-5p promoting G3BP1 and PD-L1 expression, which facilitated colorectal cancer progression and immune escape.

Introduction

Colorectal cancer (CRC) remains a serious threat to human health globally, with nearly two million new cases and approximately one million deaths caused each year (Dekker et al, 2019; Sung et al, 2021). Although the early diagnosis and clinical treatment of CRC have been greatly advanced in the last decade (Anania et al, 2019; Van Cutsem et al, 2014), it continues to be a significant burden on the individual and society. Recent molecular targeted therapy and immunotherapy are considered to be scientific breakthroughs in treatment of tumors including colorectal cancer (Franke et al, 2019; Nappi et al, 2018). Nevertheless, not all patients benefit from it. There are still unknown mechanisms in colorectal cancer development and progression. Hence, it is necessary to explore molecular mechanisms of CRC.

Long non-coding RNAs (lncRNAs) with a length of more than 200 nucleotides are a class of non-coding RNAs discovered in recent years including antisense long non-coding RNAs (antisense lncRNAs) and long intronic non-coding RNAs (lincRNAs) (Quinn & Chang, 2016; Ransohoff et al, 2018). Previously these non-coding RNAs were considered as a large class of “junk” RNA produced during transcription, until recent studies have identified that they have important biological functions (Palazzo & Koonin, 2020; Quinn &
And growing evidences support that long non-coding RNAs engage in a variety of physiological or pathological processes including organismal development and cancer (Dykes & Emanueli, 2017; Fang & Fullwood, 2016; Gutschner & Diederichs, 2012; Sanchez Calle et al, 2018; Schmitz et al, 2016). We recently reported that several lncRNAs are implicated in colorectal cancer progression (Li et al, 2021; Xu et al, 2019; Zhou et al, 2021), of which downregulated LncRNA SATB2-AS1 expression is associated with poor outcome in CRC patients. LncRNA SATB2-AS1 in the nucleus binds directly to WDR5 and GADD45A, and cis-activates SATB2 (specific AT-rich binding protein 2) transcription via regulation of histone H3 lysine 4 trimethylation (H3K4me3) deposition and DNA demethylation in the SATB2 promoter region, inhibiting colorectal cancer metastasis and affecting tumor immune cell microenvironment (Xu et al., 2019). In addition, a recent study has reported that other long non-coding RNAs localized in the cytoplasm, such as LncRNA-ATB and Linc00284, are able to bind to microRNAs (miRNAs) and cause dysregulation of miRNAs expression (Li et al, 2020; You et al, 2021). Linc00284 is highly expressed in CRC tissues and CRC cells and associated with tumor metastasis. Linc00284 regulates miR-27a/c-Met axis contributing to activation of downstream signaling pathways that result in malignant phenotypes of CRC cells (You et al., 2021).

The role of long non-coding RNAs in the development and progression of various cancers including colorectal cancer, is becoming clearer with the progressive research (Chi et al, 2019; Sanchez Calle et al., 2018), but with the increasing number of newly discovered lncRNAs, it is essential to understand precise mechanisms of these novel lncRNAs in cancer. LINC01088 located on Chromosome 4 at q21.21 is a newly discovered long non-coding RNA that has recently been investigated. A bioinformatic analysis of two GEO datasets (GSE28619 and GSE143318) identified that LINC01088 was down-regulated in liver tissues from patients with acute alcoholic hepatits compared to samples derived from donor livers (Yan et al, 2021). However, another analysis of TCGA database demonstrated that LINC01088 was significant highly-expressed risk candidate in primary lung squamous cell carcinoma compared with adjacent normal tissues and predicts poor prognosis (Liu et al, 2019). In addition, work in non-small cell lung cancer (NSCLC) indicated that LINC01088 was overexpressed in NSCLC tissues and cell lines, where LINC01088 promotes cancer cell proliferation via binding with EZH2 to repress p21 (Liu et al, 2020). Conversely, in ovarian cancer (OC), LINC01088 expression was markedly lower in OC tissues in comparison to adjacent noncancerous tissues, which is an independent predictor for overall survival (Ai et al, 2018; Zhang et al, 2018). These evidences suggest that the expression pattern of LINC01088 can be different in different types of diseases. In our previous screen, it was found to be upregulated in CRC tissues and was associated with multiple clinicopathological features in colorectal cancer patients. Herein, we investigated the role of LINC01088 in CRC with the aim of gaining a deeper understanding of colorectal cancer progression and providing potential target for CRC diagnosis and treatment.

Materials And Methods

Colorectal cancer tissues
Thirty-six cancer tissues and paired tissues were collected from CRC patients with complete pathological and clinical data who were hospitalized and treated surgically in Nanjing First Hospital from January 2015 to January 2020. Adjacent tissue was taken from a distance of more than 5 cm from cancerous tissue, and intraoperative pathological slices were pathologically confirmed to be free of cancer. There were 21 males and 15 females, aged (61.6±7.7) years, with an age range of 51-72 years. Inclusion criteria: surgically resected specimens were pathologically confirmed; all patients with colorectal cancer did not receive anti-cancer treatment such as chemotherapy, radiotherapy, immunotherapy, and targeted therapy before surgery; clinical and pathological data of CRC patients were complete; all patients gave informed consent. Besides, CRC patients with other malignant tumors, systemic infections, immunodeficiency, immune diseases, coagulation dysfunction, serious heart, liver, or kidney diseases were excluded. All patients signed informed consent form prior to surgery, and the study was approved by the Ethics Committee of Nanjing First Hospital. Table 1 lists patient characteristics.

Table 1

The clinicopathological characteristics of patients with colorectal cancer (36 cases).

| Characteristics        | Number of cases (%) |
|------------------------|---------------------|
| Age (year)             |                     |
| ≤60                    | 15 (41.67)          |
| >60                    | 21 (58.33)          |
| Gender                 |                     |
| Male                   | 17 (47.22)          |
| Female                 | 19 (52.78)          |
| TNM stage              |                     |
| +                      | 20 (55.56)          |
| −                      | 16 (44.44)          |
| Recurrence             |                     |
| No                     | 17 (47.22)          |
| Yes                    | 19 (52.78)          |
| Distant metastasis     |                     |
| M0                     | 12 (33.33)          |
| M1+M2                  | 24 (66.67)          |
Cell culture and lentivirus infection

Human colorectal cancer cell lines LoVo, Caco2, HCT116, SW480, normal human colon epithelial cells CCD841CoN, and 293T cells were obtained from the American Type Culture Collection (ATCC). LoVo cells were maintained in Ham’s F-12 culture medium with 10% FBS (Gibco) and 1% penicillin/streptomycin (P/S). Caco2, HCT116, SW480 and 293T cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) containing 10% FBS and 1% P/S. CCD841CoN cells were cultured in minimum essential medium (MEM) with 10% FBS and 1% P/S. All cells were kept in 5% CO$_2$ incubator at 37°C. LINC01088 silencing lentivirus (sh1-01088 and sh2-01088), G3BP1-overexpressing lentivirus and the control lentivirus were constructed by Shanghai GeneChem Co., Ltd. (China). CRC cells were infected with shRNA lentivirus or/and G3BP1-overexpressing lentivirus or control lentivirus at 10 MOI for 24 h following 7-days puromycin (2 μg/ml) selection. LINC01088 knockdown efficiency in the infected cells was validated by qRT-PCR.

Fluorescence in situ hybridization (FISH) assay

Fluorescence in situ hybridization (FISH) assay was conducted using RNA FISH Kit (Shanghai GenePharma Co., Ltd, China) according to the manufacturer’s protocol. All sequences used were listed below. 5’-Cy3-labeled LINC01088 homo probe 1 (5’-3’): GCAGTACTAACATCCAGTATCTGATTC; 5’-Cy3-labeled LINC01088 homo probe 2 (5’-3’): GGCGGCAGCAAGAAGCAGTTCTAAT; 5’-Cy3-labeled LINC01088 homo probe 3 (5’-3’): CTTTGCATCCATCCAGTAAAT; 5’-FAM-labeled 18S (5’-3’): CTGCCTTCCCTTGGATGGTAGCGCTTT; 5’-FAM-labeled U6 (5’-3’): TTTGCCTGTACCTCTTGC; 5’-Cy3-labeled negative control sequence (5’-3’): TGCTTTGACCGTACGCTTTT.

Transfection experiments

Small interfering RNA (siRNA) oligonucleotides including the control scrambled siRNA were ordered from Sangon Biotech (Shanghai) Co., Ltd. (China). Transient transfection of siRNA was performed with Lipofectamine™ RNAiMAX Transfection Reagent according to manufacturer’s instructions (Invitrogen, cat no.13778500). The RNA-lipid complexes were prepared, vortexed and allowed to incubate for 5 min at room temperature (RT). Next, the mixture was added into the cells followed by incubation for 24 h at 37 °C. The efficiency of siRNA interference was determined by qRT-PCR analysis 48 hours post-transfection.

Nucleo-cytoplasmic separation experiment

Nucleo-cytoplasmic fractions were isolated using PARIS™ Kit following manufacturer instructions. Briefly, adherent cells (10$^7$ cells per experiment) were harvested until they were grown to ~90% confluence, transferred to 1.5 mL microfuge tubes, and centrifuged at 180×g for 4 min at 4 °C. Next, the
supernatant was discarded. 500 μl of ice-cold Cell Fractination Buffer was added. Tubes were then placed on ice for 7 min. After centrifugation at 500×g for 4 min at 4 °C, the supernatant (cytoplasmic fraction) was transferred to a new RNase-free 1.5 mL EP tube. The pellet contained nuclear fraction. Total nuclear or cytoplasmic RNA was extracted, respectively. RNA samples were stored at -80°C or kept on ice when in use.

**Cell Counting Kit-8 (CCK-8) assay**

The cells were inoculated into 96-well dishes at a density of 3×10⁴ cells per well, and five replicate wells were set up in each analysis. After 24 h, 48 h, 72 h and 96 h of incubation respectively, CCK-8 reagent (Sigma-Aldrich, cat no.96992) with a volume fraction of 10% was added to the cells following incubation for 2 h at 37 °C. The plates were placed on a shaker for 5 min, and absorbance values were measured at 490 nm using Microplate reader (Bio-Rad). Growth curve was plotted with time as the horizontal coordinate and absorbance value as the vertical coordinate.

**Colony-formation assay**

The cells (500 cells per well) were inoculated into 6-cm culture dishes. After 2 weeks of incubation at 37 °C, cell colonies were observed and then culture medium was discarded. Adherent cells were gently washed twice with PBS (Gibco) and fixed with 4% paraformaldehyde for 15 min at RT. After elimination of fixative solution by aspiration, cell colonies were visualized by staining with 0.1% crystal violet solution (Beyotime Biotechnology, cat no.C0121, China) for 20 min at RT. The number of colonies was counted.

**Cell cycle analysis**

Cell cycle was evaluated by flow cytometry. The cells in logarithmic growth phase were digested using EDTA-free trypsin (Gibco) and washed three times with pre-chilled PBS. Thereafter, 400 μl of Binding Buffer and 10 μl of PI were added sequentially to cell suspension, following reaction for 30 min under protection from light at RT. Cycle distribution was assessed using CytoFlex Flow Cytometer (Beckman Coulter).

**Transwell migration and invasion assays**

For transwell invasion assay, Matrigel (Coming, cat no.356234) was removed from the -20°C refrigerator and placed in an ice-water mixture to dissolve. All tips and microfuge tubes used in the experiments were pre-chilled in a 4°C refrigerator. Matrigel and serum-free basal medium were mixed thoroughly at a 1:3 ratio and then added into transwell upper chambers (Costar, 8-μm pore size) for incubation for 1 h at 37°C. In addition, cell concentration was adjusted to 5×10⁵ cells/ml. 200 μl of the cell suspension was slowly added to the upper chambers and the lower chambers contained 500 μl of complete culture
medium. Twenty-four hours later, the non-invasive cells in transwell upper chamber were wiped off with wet cotton swabs. Next, the filter was fixed with 4% formaldehyde for 10 min, stained with 0.5% crystal violet for 20 min, rinsed with PBS and air-dried. The number of invasive cells was observed microscopically, and 10 randomly chosen fields of view were imaged and counted. Transwell chambers pre-coated without Matrigel were used for transwell migration assay and workflow was followed as indicated above.

**Propidium iodide (PI) staining**

After 48 h of co-culture, the cells were incubated with PI staining solution (Sigma) for 5 min. Next, PI-positive cells were observed and counted under a fluorescent microscope (Nikon), while the total number of cells was observed under white light. The percentage of PI-positive cells was calculated.

**Dual-luciferase reporter assay**

The pmiRGO dual-luciferase vectors and dual-luciferase reporter system (Promega) were used for evaluating the interaction between LINC01088 and microRNAs according to the manufacturer’s instructions. Predicted binding sites were analyzed by [https://starbase.sysu.edu.cn/starbase2/](https://starbase.sysu.edu.cn/starbase2/) and [http://starbase.sysu.edu.cn/](http://starbase.sysu.edu.cn/). The desired primer sequences were cloned into luciferase reporter plasmid as described in the instruction manual. Empty vector plasmid was as the control. 293T cells were seeded into 24-well plates and allowed to grow to 80% confluence. Reporter plasmid was co-transfected with the expression plasmid into 293T cells using Lipofectamine™ 2000 Reagent (Invitrogen). Seventy-two hours later, the proteins were extracted and Dual-Luciferase Reporter Assay System Kit (Promega) was used to test luciferase activity.

**RNA immunoprecipitation (RIP) assay**

RIP assay was performed using Imprint® RNA Immunoprecipitation Kit according to the manufacturer’s instructions (Sigma-Aldrich). Briefly, CRC cells with LINC01088 knockdown in logarithmic growth phase were harvested and resuspended using RIP Lysis Buffer, mixed thoroughly, and set aside on ice. After incubation for 30 min, the samples were centrifuged at 2500×g for 10 min at 4°C. Besides, magnetic beads conjugated anti-Ago antibody or IgG for pre-clearance were washed with RIP Wash Buffer (provided with the kit). 900 µl of RIP Immunoprecipitation Buffer was added to each tube and mixed with 100 µl supernatant following incubation overnight at 4°C with rotation. After precipitating the magnetic beads, removed the supernatant, added 500 µl of RIP Wash Buffer to wash the beads, vortexed, and then collected the precipitate. 10 µl of the cell lysate supernatant was taken as "Input" and store it temporarily at -80°C. Next, 150 µl of Proteinase K Buffer was added to the precipitate obtained in the previous step. In addition, RIP Wash Buffer, 10% SDS, and Proteinase K mixture were added to the "Input" after thawing, followed by incubation for 30 min at 55°C with shaking to digest the proteins. Total RNA for subsequent
qPCR analysis was extracted from each group according to the instructions of RNA-easy Isolation Reagent (as indicated below).

**qPCR analysis**

Total RNA was extracted from tissues or cells using RNA-easy Isolation Reagent (Vazyme Biotech Co., Ltd, cat no. R701-01) according to the instructions and the concentration of total RNA were determined using UV spectrophotometer. cDNA was reverse transcribed using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, cat no. RR047A) or miRNA 1st Strand cDNA Synthesis Kit (by stem-loop) (Vazyme Biotech Co., Ltd, cat no. MR101-01). qPCR reaction system was 20 µL: 10 µl miScript SYBR® Green Mix (QIAGEN, Dusseldorf, Germany), or 10 µl miRNA Universal SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd, cat no. MQ101-01), 2 µl cDNA, 1 µl each of forward and reverse primers, 6.0 µl ddH2O. Reaction conditions were as described below: pre-denaturation 95°C, 90 sec; denaturation 95°C, 30 sec; annealing 60°C, 30 sec; extension 72°C, 15 sec; 40 cycles in total. Gene expression was analyzed by the 2^−ΔΔCt method. GAPDH was used as an internal reference gene.

**Western blot analysis**

Tumor cells or tissues were subjected to RIPA lysis solution containing 1% PMSF and placed on ice for 20 min. The supernatant was collected by centrifugation at 12000×g for 15 min at 4°C. Protein concentration was determined by the BCA method. Appropriate amount of protein samples was mixture with 5×loading buffer and boiled for 5 min to denature the proteins. SDS-PAGE gel electrophoresis was performed to separate the proteins, and 30 µg of denatured protein samples were added to each lane. Proteins were transferred onto the PVDF membranes (Immobilon transfer membrane, Millipore Corporation) through wet transfer. Next, the membranes were blocked with bovine serum albumin diluted with TBST for 2 h at RT and incubated with primary antibody at 4°C overnight. The secondary antibody was then added and incubated for 2 h at RT. Immunoblots were visualized using Immobilon Western Chemiluminescent HRP Substrate (ECL) (Millipore). β-actin protein was used as internal control.

**Animal experiments**

To test proliferative ability of CRC cells *in vivo*, tumor cells in logarithmic growth phase were resuspended in PBS and cell concentration was adjusted to 1×10^7 cells/ml. BALB/c female nude mice of age 5 weeks and weighing about 20 g were acclimated for one week prior to experiments. 100 µl of cell suspension was subcutaneously injected into the right axilla of nude mouse (N=6 mice per group). Tumor diameters of xenograft mice were measured weekly after inoculation. Tumor volume was calculated by the formula: tumor volume = width^2×length/2 Mice were sacrificed under anesthesia 35 or 42 days after inoculation, and tumor tissues were photographed and weighed after stripping and removing the surrounding connective tissues. Besides, to measure lung metastatic capability of LINC01088-knockdown CRC cells *in
vivo, CRC cell concentration of each group was adjusted to $1 \times 10^6 \text{ cells/ml}$. 50 μl of the cell suspension was injected into the mice through the lateral tail vein of the nude mice (N=6 mice per group) and lung metastatic nodules were examined at the desired time point after deep anesthesia with isoflurane. Lung tissues were collected for HE staining. All animal experiments were conducted under the approval of Animal Care and Use Committee of Nanjing First Hospital.

**Human colorectal cancer tissue-derived organoids**

This study was approved by the Ethics Committee of Nanjing First Hospital. The patient signed informed consent form prior to surgery. Fresh colorectal cancer tissues were collected after surgical resection and maintained in ice-cold DMEM/F-12 medium with 15 mM HEPES (Gibco) within 2 hours. Tissues were cut into 1-3 mm³ pieces, washed ten times using pre-chilled PBS, and digested with Gentle Cell Dissociation Reagent (GCDR) (Stemcell Technologies, cat no.07174) for 50 min at 37°C with shaking. After centrifugation at 300×g for 5 min, the supernatant was discarded. The tissues were resuspended using DMEM/F-12 medium with 15 mM HEPES and 2% BSA (Solarbio Life Science, cat no.9048-46-8) and filtered through 70-μm strainers. The number of intestinal crypts were counted. Next, Matrigel Matrix Growth Factor Reduced (GFR), Red-free (Corning, cat no.356231) was diluted with DMEM/F-12 complete medium (as indicated above) at a 1:1 ratio and the crypts were resuspended. Matrigel containing intestinal crypts was dropped onto the center of a pre-warmed 24-well plate without medium (50 μl/well). The plate was placed in an incubator at 37°C for 10 min. Subsequently, human organoid medium IntestiCult OGM (Stemcell Technologies, cat no. A8010) was added and was changed every 2-3 days. Passaging was performed 8 days after culturing.

**Isolation, characterization and cultivation of human CD8+T cells**

Fresh blood from a healthy donor was transferred into a blood draw tube containing heparin anticoagulant and mixed upside down. PBS supplemented with 2% FBS (PBST) was added to dilute the blood at a 1:1 ratio. Subsequently, an equal volume of Lymphocyte Separation Solution (Solarbio Life Science, catalog #P8610/P8900) was added to the bottom of SepMate™-50 tube (Stemcell Technologies, catalog #15450). The diluted blood sample was aspirated and slowly spread along the wall of the tube on top of Lymphocyte Separation Solution following centrifugation at 1200×g for 10 min at room temperature. The supernatant containing mononuclear cells (MNCs) was collected, washed with PBST, and centrifuged at 300×g for 10 min at room temperature. Further, EasySep™ Human CD8+T Cell Isolation Kit (Stemcell Technologies, catalog #17953) was utilized to isolate highly purified CD8+T cells according to the manufacturer's guidance. The isolated cells were phenotypically identified by flow cytometry using anti-human CD3 antibody, clone UCHT1 (Stemcell Technologies, catalog #60011) and anti-human CD8a antibody, clone RPA-T8 (Stemcell Technologies, catalog #60022). The activity of CD8+T cells was measured by trypan blue staining. CD8+T cells were cultured with ImmunoCult™-XF T
Cell Expansion Medium (Stemcell Technologies, catalog #10981) and stimulated with ImmunoCult™ Human CD3/CD28 T Cell Activator (Stemcell Technologies, Catalog #10981).

**Bioinformatic analysis**

To explore downstream targets of LINC01088, microRNAs expression in CRC tissues were extracted from The Tumor Cancer Genome Atlas (TCGA) dataset. In addition, the relationship between tumor G3BP1 expression and immune infiltration in colorectal cancer was analyzed using Tumor IMmune Estimation Resource (TIMER) (https://cistrome.shinyapps.io/timer/) dataset (Li et al, 2016; Li et al, 2017).

**Statistical analysis**

Statistical analysis was performed using SPSS 21.0 software. Graphs were made using the GraphPad Prism 8 (GraphPad Software, San Diego, CA). All data were expressed using mean ± standard deviation and data conforming to normal distribution were compared between two groups using two-tailed Student t-test. For multi-group comparisons, statistical significance was determined by one-way analysis of variance (ANOVA) followed by Fisher's Least Significant Difference (LSD) test. Kaplan-Meier method was used to analyze the relationship between gene expression and prognosis of CRC patients. A $P<0.05$ was considered as statistically significant. *$P< 0.05$, **$P< 0.01$, ***$P< 0.001$, and NS: not significant.

**Results**

**LINC01088 is overexpressed in colorectal cancer and associated with adverse outcome**

We firstly collected 36 paired CRC tissues and adjacent normal tissues. qPCR results confirmed that LINC01088 expression was significantly elevated in colorectal cancer tumor tissues (Fig. 1A). Clinicopathological analysis showed that LINC01088 expression was associated with TNM stage, metastasis, and recurrence of patients with colorectal cancer, where LINC01088 expression was higher in Stage III/IV tissues than in Stage I/II tissues (Fig. 1A). Survival analysis revealed that CRC patients with high LINC01088 levels had worse overall survival compared with CRC patients with lower levels (Fig. 1B). Besides, LINC01088 expression was also significantly higher in CRC cells compared to normal colorectal epithelial cells and LINC01088 was located in the cytoplasm ang nucleus of tumor cells (Fig. 1C, D). Consistently, fluorescence in situ hybridization (FISH) assay further identified its localization (Fig. 1E).

**LINC01088 knockdown significantly reduces proliferative ability of human colorectal cancer cells and patient-derived organoids**
Considering high level of LINC01088 in Caco2 and LoVo cells, these two cell lines were used to construct CRC cells with stable LINC01088 knockdown by lentiviral infection. qPCR analysis validated that both shRNAs (sh1-01088 and sh2-01088) lentivirus were able to significantly reduce LINC01088 expression levels (Fig. 2A). Cell proliferation assay and colony-formation assay confirmed that proliferative and colony-forming abilities were significantly attenuated after silencing of LINC01088 in CRC cells (Fig. 2B-D). It seemed that the higher LINC01088 silencing efficiency, the lower CRC cell proliferative and colony forming capabilities. Results of cell cycle showed that LINC01088 knockdown contributed to a significant increase in the proportion of CRC cells in G0/G1 phase and a decline in G2/M phase (Fig. 2E, F). Consistent with the in vitro results, subcutaneous xenograft experiments in nude mice further proved that LINC01088 knockdown significantly inhibited CRC cell growth (Fig. 2G-I).

Human colorectal cancer organoids (Fig. 3A) were identified by immunofluorescence with anti-SOX9, Cytokeratin 20, EpCAM, Desmin, and Ki-67 antibodies (Fig. 3B). Knockdown efficiency of organoids transduced with lentivirus was measured by visualizing green fluorescence. Under white light conditions, it could be seen that the LINC01088 knockdown resulted in smaller diameter and more cellular debris (Fig. 3C), and PI staining suggested that more PI-staining positive cells were found in the organoid after LINC01088 knockdown (Fig. 3D).

**LINC01088 knockdown suppresses migration, invasion, and immune escape of CRC cells**

Next, we investigated the role of LINC01088 in migration and invasion of CRC cells. Transwell assays revealed that LINC01088 knockdown significantly reduced migration and invasion of Caco2 and LoVo cells (Fig. 4A-B). The higher LINC01088 silencing efficiency, the lower migratory and invasive capacities of the cells. This result was further reproduced in xenograft mouse model. Specially, the number and the lesion surface of the lung metastasis nodules are significantly reduced in LINC01088-knockdown groups (Fig. 4C-E). Survival analysis showed that silencing of LINC01088 significantly prolonged the survival of nude mice (Fig. 4F).

Given that tumor immune escape plays an important role in tumorigenesis and cancer progression, CRC cells were co-cultured with activated human CD8+T cells. After co-culturing with CD8+T cells for 12 hours, results of white light image and crystal violet staining showed that the density of adherent CRC cells with LINC01088 silencing was markedly reduced compared to the control, which was dependent on the number of CD8+T cells (Fig. 4G-J). It was suggested that LINC01088-knockdown CRC cells were more sensitive to CD8+T cell-mediated tumor killing relative to WT CRC cells. Similar results were observed in PI staining (Fig. 4K, L).

**LINC01088 exerts its biological activity by directly binding to intracellular microRNAs**
Long non-coding RNAs acts as competing endogenous RNAs (ceRNAs) to exhibit its activity when they localized in the cytoplasm. In this study, LINC01088 was mainly localized in the cytoplasm. Hence, we suspected that LINC01088 exerts its biological function via IncRNA-microRNA (miRNA)-messenger RNA (mRNA)-ceRNA networks. Therefore, microRNAs (miRNAs) that were significantly downregulated in colorectal cancer and predicted to bind directly to LINC01088 were analyzed by bioinformatics methods. A list of 35 miRNAs was obtained (Fig. 5A). And we further confirmed that 20 miRNAs were able to be directly down-regulated by LINC01088 in colorectal cancer by RIP assay (Fig. 5B). The expression levels of these 20 miRNAs were examined in LoVo cells with LINC01088 knockdown, and miRNAs that were up-regulated after silencing LINC01088 were taken into consideration (Fig. 5C, D). Finally, 9 miRNAs were selected as targets for further analysis (Fig. 5E). Next, specific inhibitors of miRNAs were transfected into LINC01088-knockdown CRC cells to determine the primary downstream targets. Since miR-548b-5p and miR-548c-5p inhibitors could reverse phenotypes induced by LINC01088 knockdown, miR-548b-5p and miR-548c-5p were identified as potentially important downstream targets of LINC01088 (Fig. 6A-D). Dual-luciferase assay experiments further confirmed that miR-548b-5p and miR-548c-5p were indeed direct targets of LINC01088 (Fig. 6E-H).

**LINC01088 targets miR-548b-5p and miR-548c-5p, regulating G3BP1 expression**

MicroRNAs usually regulate mRNA expression by directly binding to them. By bioinformatics analysis, we searched for downstream target genes jointly predicted by miR-548b-5p and miR-548c-5p in multiple databases, respectively. miR-548b-5p had 9 target genes and miR-548c-5p had 41 target genes. By taking the intersection of these two sets of genes, we obtained a list of genes including **BRWD1, G3BP1, CCDC47, DDIT4, RORA, UE4A, LY75, TRA2B** (Fig. 7A). Combining survival and expression analysis in colorectal cancer, we concluded that G3BP1 may be a key downstream target of LINC01088/miRNAs axis. Ras-GTPase-activating protein SH3 domain-binding protein 1 (G3BP1) was proved to be overexpressed in a variety of cancers and targeting G3BP1 exhibited anti-tumor activity (Zhang et al, 2019). Further analysis from TCGA and TIMER databases revealed that in most human cancers including colorectal cancer G3BP1 expression is upregulated and associated with immune infiltration in colorectal cancer (Fig. S1). Next, we firstly explored G3BP1 protein expression in LINC01088-knockdown LoVo cells. Western blotting analysis showed that treatment with miR-548b-5p/ miR-548c-5p mimics contributed to a significant decrease in G3BP1 protein levels in CRC cells (Fig. 7B, C). Next, predicted binding sites between LINC01088 and miR-548b-5p (Fig. 7D), LINC01088 and miR-548c-5p (Fig. 7E) were detected by dual-luciferase reporter assay. Data revealed that three binding sites between LINC01088 and miR-548b-5p are available (Fig. 7F) and two binding sites between LINC01088 and miR-548c-5p were identified (Fig. 7G).
LINC01088/microRNAs/G3BP1/PD-L1 axis promotes colorectal cancer progression

As previously described, our results suggested that LINC01088 knockdown repressed CRC cell proliferation, migration, and invasion. Subsequently, we implemented rescue experiments to verify effects of LINC01088/microRNAs/G3BP1 axis on malignant phenotypes of colorectal cancer. LINC01088 knockdown resulted in decreased G3BP1 expression at the transcriptional nor the translational levels (Fig. 8A, B), reduced cell viability, migratory and invasive capabilities, and weakened CD8+ T cell-mediated tumor killing activity, whereas overexpression of G3BP1 could partially reverse G3BP1 expression and phenotypes caused by LINC01088 knockdown in Caco2 cells (Fig. 9C-G). Besides, when LINC01088-knockdown Caco2 cells were co-cultured with activated CD8+ T cells for 12 h, G3BP1 overexpression reduced lactate dehydrogenase (LDH) levels that were significantly elevated as LINC01088 knockdown (Fig. S2). Specially, LINC01088 knockdown caused a significant decrease in PD-L1 mRNA expression and cell-surface PD-L1 expression in Caco2 cells (Fig. 8H, I). Further, tumor xenograft in nude mice revealed that G3BP1 overexpression restored LINC01088 knockdown-induced suppression of cell proliferation (Fig. 8J, K). Remarkably, LINC01088 knockdown downregulated PD-L1 expression, while G3BP1 overexpression could reverse PD-L1 expression in xenograft tumors. It implied that G3BP1 regulated PD-L1 expression in CRC (Fig. 8L). These findings suggested that upregulation of LINC01088 promotes malignant phenotypes and immune escape of colorectal cancer by regulating microRNAs/G3BP1/PD-L1 axis (Fig. 9).

Discussion

Colorectal cancer is a great threat to human health. Explorations of the pathogenesis of colorectal cancer as well as clinical treatments have achieved significant progress in recent decades (Dekker et al., 2019; Nguyen et al, 2020). However, considering that long-term survival and quality of life of colorectal cancer patients have been dismal, further researches on specific mechanisms are deserved to find novel therapeutic strategies. In the present study, we found that LINC01088 was significantly upregulated in colorectal cancer tissues and LINC01088 promoted colorectal cancer progression by mediating microRNAs/G3BP1 axis.

LincRNA is one of the long non-coding RNAs (Ransohoff et al., 2018), and mounting evidence has confirmed that dysregulated LincRNA expression in tumor tissues plays an important role in multiple disease processes (Ransohoff et al., 2018; Ulitsky & Bartel, 2013). Previous studies found that LINC01088 inhibited trophoblast cell function and led to recurrent miscarriages by activating MAPK signaling pathway (Zhao et al, 2021). Work in lung squamous cell carcinoma (LSqCC) demonstrated that through analyzing transcriptome profiling of 1771 lincRNAs in the TCGA database from 549 samples of 501 LSqCC patients, 10 lincRNAs including LINC01088 were confirmed to be significant highly-expressed risk candidates and were associated with poor prognosis (Liu et al., 2019). Another recent study has identified that LINC01088 directly binds to EZH2 inhibiting the expression of p21, a well-known oncogenic factor,
thereby suppressing proliferation of lung cancer cells (Liu et al., 2020). Similarly, our data demonstrated that elevated LINC01088 expression facilitated proliferation of colorectal cancer cells, contributing to colorectal cancer progression. Mechanistically, LINC01088 regulated the proliferative capacity of colorectal cancer cells by adsorbing a series of intracellular miRNAs acting as a pro-cancer factor. In contrary, there is also evidence that LINC01088 expression levels are significantly lower in ovarian cancer tissues relative to ovarian epithelial tissues (Zhang et al., 2018) and low LINC01088 expression is correlated with FIGO staging, grade and metastasis (Ai et al., 2018), where LINC01088/miR-24-1-5p/p21-activated kinase 4 (PAK4) axis may be responsible for the oncogenic role of LINC01088 in ovarian epithelium (Zhang et al., 2018). LINC01088 function is differential in different tissues.

Invasive capacity (Novikov et al, 2021; Yeung & Yang, 2017) and immune escape (Liu & Cao, 2016; Picard et al, 2020) of cells are important factors for tumor development. Our study confirmed that LINC01088 knockdown significantly reduced migratory and invasive capabilities and facilitated immune escape of colorectal cancer cells. Specifically, LINC01088 positively regulates G3BP1 expression by directly binding to and negatively regulating miR-548b-5p and miR-548c-5p, two oncogenic factors in a variety of cancers including colorectal cancers (Pan et al, 2016; Wang et al, 2020; Xu et al, 2020). G3BP1 is an oncogenic gene and targeting G3BP1 may be a promising antitumor strategy (Zhang et al., 2019). Our study is the first time to confirm the correlation between miR-548b-5p, miR-548c-5p and G3BP1. Restoration of G3BP1 levels reversed phenotypes associated with malignancy including proliferation, migration and immune escape LINC01088 knockdown CRC cells. This may provide a potential target for colorectal cancer treatment.

In summary, our findings have confirmed that LINC01088 is significantly upregulated in colorectal cancer tissues, and directly binds to miR-548b-5P and miR-548c-5P to regulate G3BP1 and PD-L1 expression, promoting colorectal cancer progression. LINC01088 exhibited tumor-promoting property. This work provides novel insights into the pathogenesis of colorectal cancer.

Declarations

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Ethics Statements

All patients signed informed consent form prior to surgery, and the study was approved by the Ethics Committee of Nanjing First Hospital. All animal experiments were conducted under the approval of Animal Care and Use Committee of Nanjing First Hospital.
Data Availability Statement

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

Conflicts of Interest

The authors declare no conflict of interest.

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Figures

Figure 1

LINC01088 expression in tumor tissues of patients with colorectal cancer and its subcellular localization. (A) LINC01088 expression levels in tumor tissues and adjacent normal tissues of patients with colorectal cancer. Association analysis of LINC01088 expression and the patients’ clinical characteristics including tumor-node-metastasis stages, distant metastasis and recurrence. (B) Survival analysis based on
LINC01088 expression levels. (C) LINC01088 expression in human colorectal cancer cell lines and colonic normal epithelial cells. (D) Nucleocytoplasmic distribution of LINC01088 in CRC cells. (E) Subcellular localization of LINC01088 in cells and tissues was tested by FISH assay. Data were expressed using mean ± standard deviation. Comparison between two groups using two-tailed Student t-test. For multi-group comparisons, statistical significance was determined by one-way ANOVA followed by Fisher’s LSD test. Kaplan-Meier method was used to analyze the relationship between gene expression and prognosis of CRC patients. *P<0.05, **P<0.01, ***P<0.001. TNM, tumor-node-metastasis; M, metastasis; R, recurrence; CRC, colorectal cancer; FISH, RNA-fluorescence in situ hybridization.

Figure 2

Effects of LINC01088 knockdown on CRC cell proliferation in vitro and in vivo. (A) LINC01088 knockdown in CRC cells was achieved by lentivirus-mediated shRNA interference. (B) Cell viability of LINC01088-knockdown CRC cells at different time points was measured by CCK-8 assay. (C, D) Colony-forming ability of LINC01088-knockdown (C) Caco2 and (D) LoVo cells was evaluated by colony formation assay after culturing for 14 consecutive days. (E, F) Cell cycle distribution of LINC01088-knockdown (E) Caco2 and (F) LoVo cells was tested by flow cytometry. (G) Tumor growth of LINC01088-knockdown xenograft tumors. (H) Image of xenograft tumors at 42 days after inoculation. (I) Tumor volume of LINC01088-knockdown xenograft tumors at 42 days after inoculation. N=6 mice per group. Comparisons among groups were analyzed by one-way ANOVA followed by Fisher’s LSD test. *P<0.05, **P<0.01, ***P<0.001. CRC, colorectal cancer.

Figure 3

Cultivation, identification and lentivirus infection of human colorectal cancer organoids. (A) Organoids at P0 and P1. (B) Immunofluorescence staining for organoids using anti-SOX9, Cytokeratin 20, EpCAM, Desmin, and Ki-67 antibodies. (C) Organoids at P1 were infected with LINC01088-knockdown and control lentivirus. (D) Organoids infected with lentivirus were stained with PI (1 μg/ml) for 5 min. Green indicates GFP fluorescence; and red, PI fluorescence.

Figure 4

Effects of LINC01088 knockdown on the migration and invasion, lung metastatic ability of CRC cells in vitro and in vivo. (A, B) Migratory and invasive capabilities of LINC01088 knockdown (A) Caco2 and (B) LoVo cells were tested by transwell assays. (C) Schematic illustration of animal experiments. (D) Representative images and quantification of lung metastatic nodules in xenograft mouse model. (E) HE
staining of lung metastatic nodules. (F) Survival curves of CRC-bearing nude mice. (G, H) After coculturing for 12 h, LINC01088-knockdown CRC cells and CD8+ T cells were imaged under the bright field. (I, J) After coculturing for 12 h, surviving CRC cells were stained with crystal violet. (K, L) After coculturing for 12 h, apoptotic CRC cells were stained by PI. Data were expressed using mean ± standard deviation. Comparison between two groups using two-tailed Student t-test. For multi-group comparisons, statistical significance was determined by one-way ANOVA followed by Fisher’s LSD test. *P<0.05, **P<0.01, ***P<0.001. HE, haematoxylin-eosin staining; PI, propidium iodide. CRC, colorectal cancer.

Figure 5

Determination of the downstream targets of LINC01088 in colorectal cancer. (A) Screening process of the downstream targets of LINC01088. (B) RIP assay was performed to validate the relationship between microRNA and Linc01088 in Caco2 cells. (C, D) The expression levels of 20 microRNAs in (C) sh1-LINC01088 Caco2 cells and (D) sh2-LINC01088 Caco2 cells. (E) Nine differentially expressed microRNAs that interacted directly with LINC01088 in Caco2 cells. Data were expressed using mean ± standard deviation. Comparison between two groups using two-tailed Student t-test. For multi-group comparisons, statistical significance was determined by one-way ANOVA followed by Fisher’s LSD test. *P<0.05. RIP, RNA immunoprecipitation assay.

Figure 6

miR-548b-5p and miR-548c-5p are the main direct downstream targets of LINC01088 in colorectal cancer. (A) Cell viability of LINC01088-knockdown Caco2 cells transfected with microRNA inhibitor for 48 h. (B) Representative images and statistical analysis of the migration and invasion of LINC01088-knockdown Caco2 cells transfected with microRNA inhibitor. (C) After coculturing for 12 h, LINC01088-knockdown Caco2 cells transfected with microRNA inhibitor were stained with crystal-violet. (D) Two microRNAs were determined according to the Venn diagram. (E, G) Predicted binding sites of (E) miR-548b-5p or (G) miR-548c-5p on LINC01088 sequence. (F, H) The interaction between LINC01088 and (F) miR-548b-5p or (H) miR-548c-5p was determined by dual-luciferase reporter system analysis. Data were expressed using mean ± standard deviation. Comparisons among groups was analyzed by one-way ANOVA followed by Fisher’s LSD test. *P<0.05, **P<0.01, ***P<0.001, NS, not significant.
G3BP1 is the common direct downstream target of miR-548b-5p and miR-548c-5p in colorectal cancer. 

(A) Screening process of the common downstream targets of miR-548b-5p and miR-548c-5p. (B, C) G3BP1 protein expression in Caco2 cells after transfection with (B) miR-548b-5p or (C) miR-548c-5p mimics for 48 h. (D, E) Predicted binding sites between G3BP1 mRNA and (D) miR-548b-5p or (E) miR-548c-5p. (F, G) The interaction between G3BP1 mRNA and (F) miR-548b-5p or (G) miR-548c-5p was determined by dual-luciferase reporter system analysis. Data were expressed using mean ± standard deviation. Comparison between two groups using two-tailed Student t-test. Comparisons among groups was analyzed by one-way ANOVA followed by Fisher’s LSD test. *P<0.05, **P<0.01, ***P<0.001, NS, not significant.

Figure 8

Effects of LINC01088/microRNAs/G3BP1 axis on colorectal cancer. (A, B) The (A) mRNA and (B) protein expression levels of G3BP1 in LINC01088-knockdown Caco2 cells. (C, D) The (C) mRNA and (D) protein expression levels of G3BP1 in LINC01088-knockdown Caco2 cells infected with or without G3BP1-overexpressing lentivirus. (E) Cell viability was evaluated by CCK-8 assay. (F) Cell migratory and invasive capabilities were tested by transwell assays. (G) After co-culturing with CD8+T cells for 12 h, surviving Caco2 cells were stained with crystal-violet. (H) PD-L1 mRNA expression in LINC01088-knockdown CRC cells. (I) PD-L1 cell-surface expression was assessed by flow cytometry. (J) Growth curves of xenograft tumors. (K) Tumor volume of xenograft tumors at 36 days after inoculation. (L) PD-L1 mRNA expression in xenograft tumors (n=3 per group). Data were expressed using mean ± standard deviation. Comparison between two groups using two-tailed Student t-test. Comparisons among groups was analyzed by one-way ANOVA followed by Fisher’s LSD test. **P<0.01, ***P<0.001.

Figure 9

Effects of LINC01088/microRNAs/G3BP1 axis on colorectal cancer. (A, B) The (A) mRNA and (B) protein expression levels of G3BP1 in LINC01088-knockdown Caco2 cells. (C, D) The (C) mRNA and (D) protein expression levels of G3BP1 in LINC01088-knockdown Caco2 cells infected with or without G3BP1-overexpressing lentivirus. (E) Cell viability was evaluated by CCK-8 assay. (F) Cell migratory and invasive capabilities were tested by transwell assays. (G) After co-culturing with CD8+T cells for 12 h, surviving Caco2 cells were stained with crystal-violet. (H) PD-L1 mRNA expression in LINC01088-knockdown CRC cells. (I) PD-L1 cell-surface expression was assessed by flow cytometry. (J) Growth curves of xenograft tumors. (K) Tumor volume of xenograft tumors at 36 days after inoculation. (L) PD-L1 mRNA expression in xenograft tumors (n=3 per group).
in xenograft tumors (n=3 per group). Data were expressed using mean ± standard deviation. Comparison between two groups using two-tailed Student t-test. Comparisons among groups was analyzed by one-way ANOVA followed by Fisher’s LSD test. **$P<0.01$, ***$P<0.001$.

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