Long Noncoding RNA LINC01133 Promotes the Malignant Behaviors of Renal Cell Carcinoma by Regulating the miR-30b-5p/Rab3D Axis

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Abstract

Renal cell carcinoma (RCC) is the most common type of kidney cancer with rising incidence. Long noncoding RNA (lncRNA) LINC01133 is a novel lncRNA that is involved in the development of several types of cancers. However, the role of LINC01133 in RCC has not been reported. Thus, in this study, we investigated the functions of LINC01133 in RCC. The qualitative real-time polymerase chain reaction analysis was performed to examine the levels of LINC01133 in RCC tissues and adjacent tissues, as well as RCC cell lines. The results showed that LINC01133 was highly expressed in RCC tissue specimens and cell lines. Downregulation of LINC01133 significantly inhibited the proliferation, migration, and invasion of RCC cells. Further mechanistic investigations proved that LINC01133 directly interacted with microRNA (miR)-30b-5p and regulated the miR-30b-5p expression in RCC cell lines. Moreover, miR-30b-5p exhibited tumor-suppressive activity in RCC cell lines, which was mediated by targeting Ras-related protein Rab-3D (Rab3D). *In vivo* study showed that LINC01133 knockdown suppressed tumor growth in the nude mice. Taken together, these findings indicated that LINC01133 might be an oncogene in RCC through regulation of the miR-30b-5p/Rab3D axis. Thus, LINC01133 might serve as a potential therapeutic target for the treatment of RCC.

Keywords

renal cell carcinoma, long noncoding RNA LINC01133, miR-30b-5p, Ras-related protein Rab-3D

Introduction

Renal cell carcinoma (RCC) is the most common lethal genitourinary cancer originating in the lining of the proximal convoluted tubule[1]. Although the management of RCC has been developed in the past decades, new therapeutic options improving the life expectancy of patients with advanced RCC are still needed[2]. It has been demonstrated that gene alterations are closely associated with increased risk and development of RCC[3]. Better understanding of these alterations can be helpful for the development of targeted therapeutic approaches[3].

Long noncoding RNAs (lncRNAs) are a type of RNA that are not able to translate into proteins with lengths exceeding 200 nucleotides[4]. In recent years, more and more researchers have focused on the biological function of lncRNAs[4]. Based on the literatures, lncRNAs are extensively reported to be involved in the regulation of gene transcription[5]. A handful of studies have implicated lncRNAs in a variety of diseases, such as cancers[6]. Aberrant expressions of lncRNAs drive cancer phenotypes through interaction with other cellular macromolecules, making them the targets for effective diagnosis and therapeutic approach in the prevention and treatment of cancers[7].

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LINC01133 is a novel lncRNA that has been proven to be involved in the tumorigenesis of several types of cancers, such as lung squamous cell cancer[8], non-small-cell lung cancer[9], colorectal cancer[10], pancreatic ductal adenocarcinoma[11], and osteosarcoma[12]. LINC01133 exhibits either suppressive or promotive activity on tumorigenesis and may be a prognostic marker in these cancers. However, the role of LINC01133 in RCC has not been investigated. In the present study, we explored the function of LINC01133 in RCC both *in vitro* and *in vivo*.

Materials and Methods

Clinical Tissues and Cell Culture

A total of 34 paired RCC issues and correlatively adjacent nontumor tissues were obtained from RCC patients who underwent resection surgery at The Second Affiliated Hospital of Medical School, Xi'an Jiaotong University. The clinical tissues were immediately frozen in liquid nitrogen and stored at –80°C. The usage of clinical tissues samples in the present study was approved by the Ethics Committee of The Second Affiliated Hospital of Medical School, Xi'an Jiaotong University. Informed consent was obtained from each patient prior to the study.

The human renal proximal tubular epithelial cell line (HKC), and human RCC cell lines ACHIN, A498, SN12PM6, and 786-O cells were purchased from ATCC (Manassas, VA, USA). The cell lines were cultured in RPMI1640 medium or Dulbecco's modified Eagle medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% (v/v) fetal bovine serum (FBS; Gibco) and 1% antibiotics (100 µg/ml streptomycin and 100 U/ml penicillin). The cells were maintained in a humidified atmosphere containing 5% CO_2 at 37°C.

Cell Transfection

The lentivirus containing specific short hairpin RNA (shRNA) against LINC01133 (LV-LINC01133) and control lentivirus (LV-NC) were packaged by GeneChem (Shanghai, China). The LV-LINC01133 or LV-NC was then used to infect RCC cells. The expressions of LINC01133 were confirmed by qualitative real-time polymerase chain reaction (qRT-PCR) assay after selection of stable cells.

786-O cells were transfected with miR-30b-5p mimics/negative control (miR-NC) and/or Rab3D-overexpressing vector/empty vector, which were obtained from RiboBio (Guangzhou, China). The transfection was performed by using Lipofectamine 3000 (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Cell Proliferation Assay

786-O cells with different infections were seeded in 96-well plates at 1×10^3 cells per well. Following 0, 24, 48, 72, or 96 h incubation, 10 µl of 5 mg/ml MTT (Sigma, St. Louis, MO,
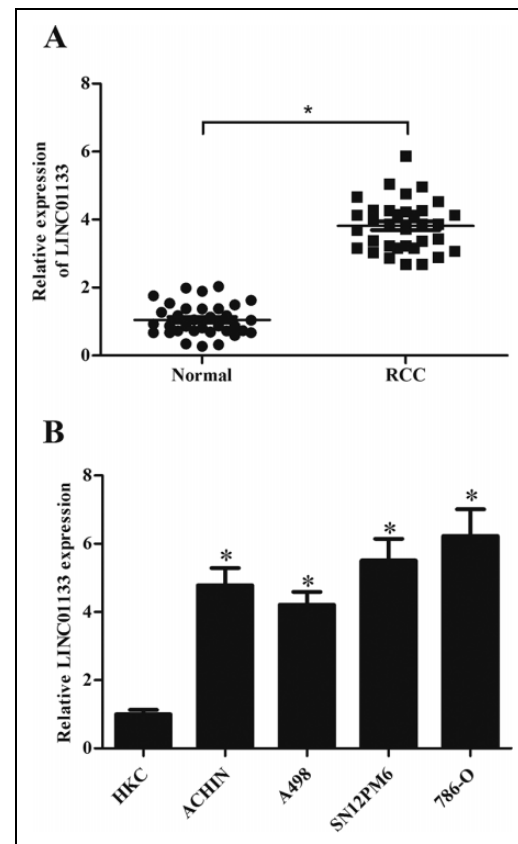

Fig. 1. LINC01133 expression was elevated in RCC tissue specimens and cell lines. (A) The qualitative real-time polymerase chain reaction analysis was performed to examine the levels of LINC01133 in 34 paired RCC tissues and adjacent tissues. (B) LINC01133 expressions in control HKC cells and human RCC cell lines including ACHIN, A498, SN12PM6, and 786-O cells. *$P < 0.05$. HKC: human renal proximal tubular epithelial cell line; RCC: renal cell carcinoma.

USA) was added to each well. After incubation for 4 h at 37°C in the dark, the supernatant was discarded, and 100 µl dimethyl sulfoxide was added to solubilize the formazan crystals. Finally, the absorbance values at 490 nm were detected with a microplate reader (Bio-Tek, Winooski, VT, USA).

Cell Invasion Assay

Cell invasion was examined by using a Matrigel-coated Transwell chamber (8-µm-pore size; Corning Inc., Corning, NY, USA). Briefly, RCC cells resuspended in serum-free medium were seeded into the upper chamber at a density of 5×10^4 cells, while 600 µl DMEM containing 10% FBS was added to the lower chamber. After incubation for 24 h, cells remaining on the upper surface of the inserts were wiped off using cotton swabs. Cells moving into the lower side of the inserts were fixed with 5% polytetrafluoroethylene and subsequently stained with crystal violet. Five fields were imaged
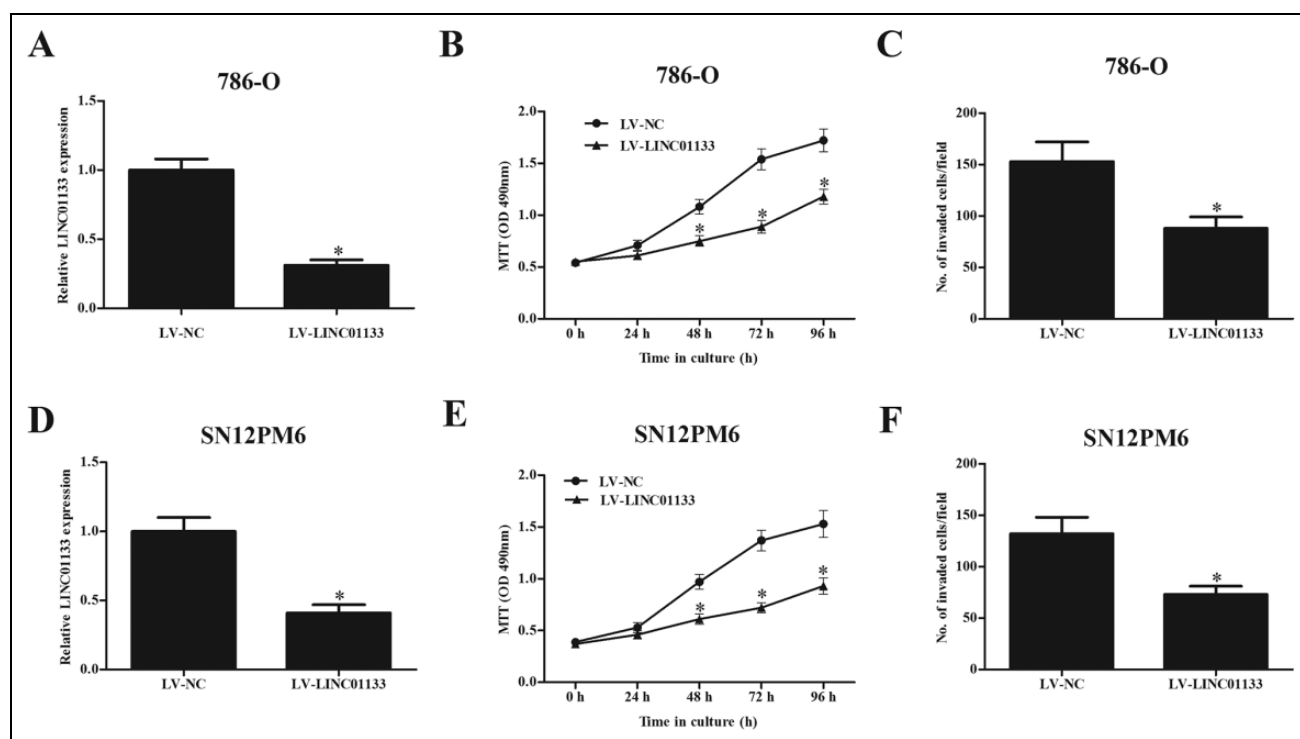
Fig. 2. Knockdown of LINC01133 inhibited the proliferation and invasion of RCC cells. (A, D) 786-O and SN12PM6 cells were infected with LV-LINC01133 or LV-NC. The stable cells were confirmed by qualitative real-time polymerase chain reaction. (B, E) MTT assay was used to detect cell proliferation of 786-O and SN12PM6 cells, respectively. (C, F) Transwell assay was carried out to assess cell invasion in 786-O and SN12PM6 cells, respectively. *P < 0.05.
LV-NC: control lentivirus.
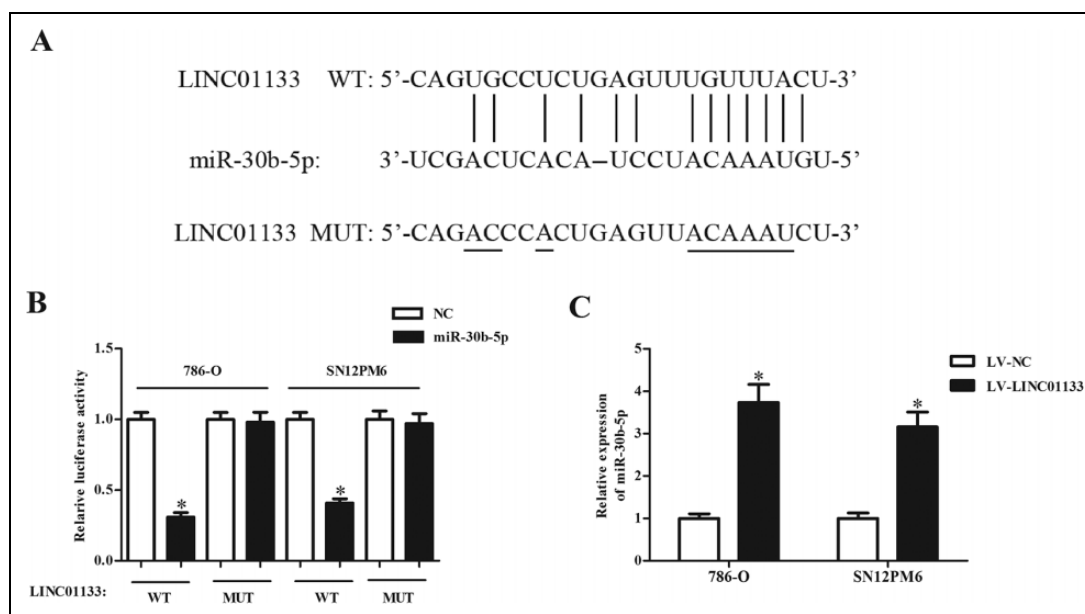
Fig. 3. LINC01133 acted as a sponge of miR-30b-5p in renal cell carcinoma cells. (A) Predicted results of the interaction between LINC01133 and miR-30b-5p. (B) Luciferase reporter assay was performed to confirm the interaction between LINC01133 and miR-30b-5p in 786-O and SN12PM6 cells, respectively. *P < 0.05. (C) Effect of LINC01133 knockdown on miR-30b-5p expression in 786-O and SN12PM6 cells, respectively. *P < 0.05.
LV-NC: control lentivirus; WT: wild type.

at a magnification of ×100 and the cell numbers were counted with a light microscope (Olympus, Tokyo, Japan).

Qualitative Real-time Polymerase Chain Reaction

Total RNAs of the collected clinical tissues and cell lines were extracted using the TRIzol reagent (Sigma-Aldrich). Then the cDNA was synthesized using the First Strand cDNA Synthesis Kit (Sigma) following the manufacturer's instructions. Afterwards, the PCR was performed using KiCqStart SYBR Green qPCR ReadyMix (Sigma) on an ABI PRISM 7900 real-time system (Applied Biosystems, Foster, CA, USA). The detection of miR-30b-5p expression was performed using All-in-One miRNA qRT-PCR Detection Kit (Gene Copoeia, Rockville, MD, USA). β-actin or U6 was used as an internal reference. Comparative Ct method was used for the quantification of the relative expressions of LINC01133 and miR-30b-5p.

Western Blot

Total protein was extracted from RCC cells using RIPA Lysis (Beyotime Biotechnology, Shanghai, China) and then the protein concentration was quantified using bicinchoninic acid method (Beyotime). Then equal amounts of proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and the following western blot analysis as previously described[13]. The primary antibodies against Ras-related protein Rab-3D (Rab3D) and β-actin, as well the horseradish peroxidase-linked secondary antibody were obtained from Abcam (Cambridge, MA, USA). The protein bands were analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA).

Target Prediction

The potential target miRNAs of LINC01133 and the target genes of miR-30b-5p were predicted using online software miRbase and TargetScan.

Luciferase Reporter Assay

The pGL/Luc-LINC01133-WT/pGL/Luc-LINC01133-MUT and pGL/Luc-Rab3D-WT/pGL/Luc-Rab3D-MUT vectors were constructed and then co-transfected with miR-30b-5p mimics or miR-NC into 786-O and SN12PM6 cells. After 48 h, the cell lysates were prepared, and the luciferase activity was measured using Dual-Luciferase Reporter System (Promega, Madison, WI, USA).

Tumor Growth in Nude Mice

Female nude BALB/7 mice (6 weeks old; Beijing Weitong Lihua Laboratory Animal Co, Ltd. Beijing, China) were randomly divided into two groups. For the xenograft implantation experiment, approximately 2×10^6 LV-LINC01133-infected 786-O cells and LV-NC-infected 786-O cells were
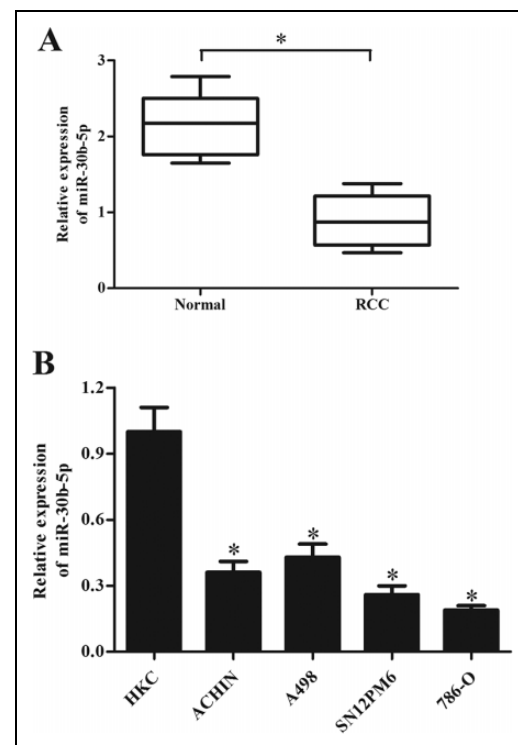

Fig. 4. The expression of miR-30b-5p was decreased in RCC tissue specimens and cells. (A) The expression of miR-30b-5p in RCC tissues was measured using qualitative real-time polymerase chain reaction assay. (B) miR-30b-5p expressions in control HKC cells and human RCC cell lines including ACHIN, A498, SN12PM6, and 786-O cells. *$P < 0.05$.
HKC: human renal proximal tubular epithelial cell line; RCC: renal cell carcinoma.

subcutaneously implanted into both flanks of mice in control group ($n = 5$) and shRNA-LINC00858 group ($n = 5$), respectively. Tumor growth in nude mice was monitored once a week. The tumor volume was calculated using the formula: tumor volume = $0.5 \times$ length $\times$ width^2. After 4 weeks, all mice were sacrificed by intraperitoneal injection of sodium pentobarbital, and tumors were collected for the determination of weight and volume. All animal experiments were approved by the Animal Care and Use Committee of The Second Affiliated Hospital of Medical School, Xi'an Jiaotong University (Xi'an, China) and conducted in accordance with the Institutional Animal Care and Use Committee guidelines.

Statistical Analysis

Data were represented as the means $\pm$ standard deviation. The statistical analysis was conducted using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA). The comparisons among multiple groups were performed using one-way analysis of variance, while the comparisons between two groups were carried out using two-tailed Student's *t*-tests. A *P*-value less than 0.05 was considered to be statistically significant (*$P < 0.05$).
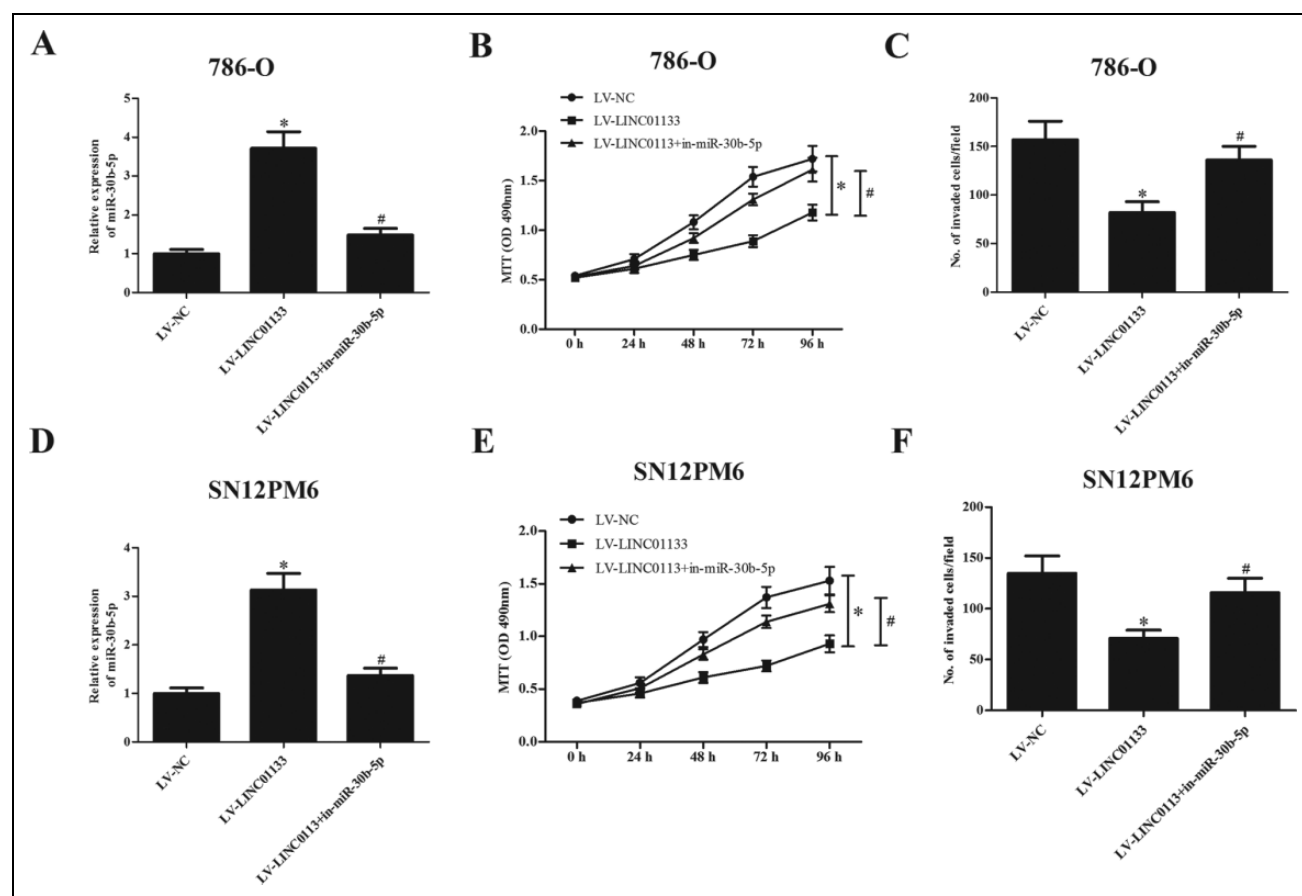
Fig. 5. Inhibition of miR-30b-5p reversed the inhibitory effects of LINC01133 knockdown on RCC cell proliferation and invasion. RCC cells were co-transfected with LV-LINC01133 and miR-30b-5p inhibitor. (A, D) The expression of miR-30b-5p was measured using qualitative real-time polymerase chain reaction assay in 786-O and SN12PM6 cells, respectively. (B, E) MTT assay was used to detect cell proliferation of 786-O and SN12PM6 cells, respectively. (C, F) Transwell assay was carried out to assess cell invasion in 786-O and SN12PM6 cells, respectively. *$P < 0.05$ versus LV-NC, #$P < 0.05$ versus LV- LINC01133.
LV-NC: control lentivirus.

Results

LINC01133 Was Highly Expressed in RCC Tissue Specimens and Cells

The qRT-PCR was performed to examine the levels of LINC01133 in 34 paired RCC tissues and adjacent nontumor tissues. The results showed that LINC01133 expression was significantly increased in RCC tissues compared with adjacent nontumor tissues (Fig. 1A). Then the LINC01133 expressions in human RCC cell lines (ACHIN, A498, SN12PM6, and 786-O cells) and control cells (HKC) were determined. As shown in Fig. 1B, LINC01133 expressions in human RCC cell lines were markedly higher than that in the HKC. The results indicated that LINC01133 might play an important role in the development and progression of RCC.

Downregulation of LINC01133 Inhibited the Proliferation and Invasion of RCC Cells

In order to investigate the function of LINC01133, 786-O and SN12PM6 cells were infected with LV-LINC01133 to knock down LINC01133, respectively. The knockdown efficiency was confirmed by qRT-PCR, as presented in Fig. 2A, D. MTT assay revealed that knockdown of LINC01133 significantly suppressed the proliferation of 786-O and SN12PM6 cells, respectively (Fig. 2B, E). Moreover, cell invasion was dramatically suppressed by LINC01133 knockdown in 786-O and SN12PM6 cells (Fig. 2C, F).

LINC01133 Targeted miR-30b-5p in RCC Cells

We used the online software TargetScan to predict that the miRNAs interacted with LINC01133, and found that miR-30b-5p could bind to complementary sequences in LINC01133 (Fig. 3A). Luciferase reporter assay denoted that the luciferase activities in 786-O and SN12PM6 cells were markedly decreased after co-transfection with pGL/Luc-LINC01133-WT and miR-30b-5p mimics, respectively (Fig. 3B). Furthermore, knockdown of LINC01133 significantly increased miR-30b-5p expression in 786-O and SN12PM6 cells (Fig. 3C).
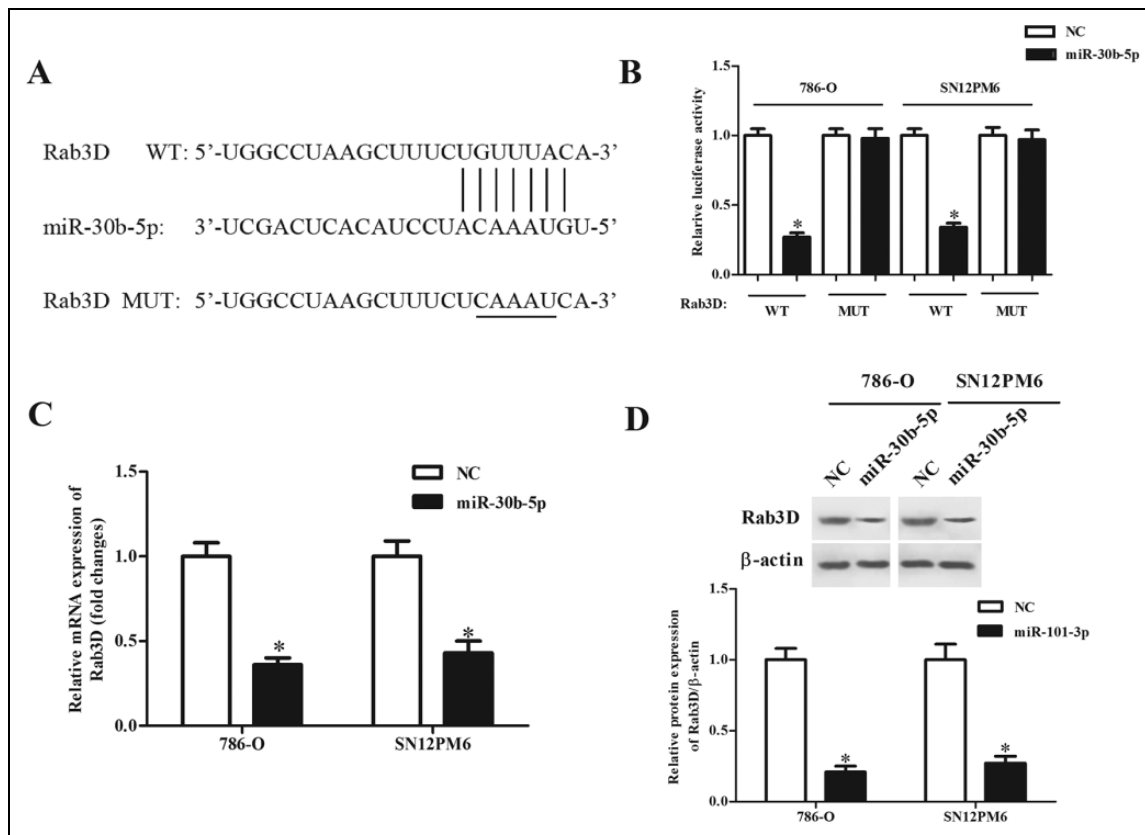
Fig. 6. MiR-30b-5p targets Rab3D in RCC cells. (A) Predicted binding sites between miR-30b-5p and Rab3D. (B) Luciferase reporter assay was conducted to confirm the interaction between miR-30b-5p and Rab3D in 786-O and SN12PM6 cells. (C, D) Qualitative real-time polymerase chain reaction and western blot analysis were performed to examine the effect of miR-30b-5p on Rab3D expression in 786-O and SN12PM6 cells. *$P < 0.05$.
NC: negative control; WT: wild type.

The Expression of miR-30b-5p Was Decreased in RCC Tissue Specimens and Cells

To examine the role of miR-30b-5p in RCC, we detected the expression of miR-30b-5p in RCC tissues and cell lines using qRT-PCR assay. We found that miR-30b-5p expression was significantly downregulated in RCC tissues compared with adjacent nontumor tissues (Fig. 4A). In addition, miR-30b-5p expressions in human RCC cell lines were markedly lower than that in the HKC (Fig. 4B).

Inhibition of miR-30b-5p Reversed the Inhibitory Effects of LINC01133 Knockdown on RCC Cell Proliferation and Invasion

Rescue experiments were conducted to examine whether LINC01133 exerted its function through miR-30b-5p in RCC cells. We detected the expression of miR-30b-5p and found that miR-30b-5p inhibitor significantly decreased LINC01133 knockdown-induced miR-30b-5p expression in 786-O and SN12PM6 cells, respectively (Fig. 5A, D). In

addition, the results of MTT assay showed that transfection with miR-30b-5p inhibitor could reverse the inhibitory effect of LINC01133 knockdown on cell proliferation in 786-O and SN12PM6 cells, respectively (Fig. 5B, E). Similarly, the inhibitory effect of LINC01133 knockdown on cell invasion was partially reversed by miR-30b-5p inhibitor in 786-O and SN12PM6 cells, respectively (Fig. 5C, F).

MiR-30b-5p Targets Rab3D in RCC Cells

Subsequently, we searched the online software to find the target genes of miR-30b-5p. As presented in Fig. 6A, Rab3D might be a target gene of miR-30b-5p. In addition, results from luciferase reporter assay showed that miR-30b-5p mimics led to marked decreases in luciferase activities in Rab3D-WT reporter compared with control group, but had no obvious effect on the luciferase activity in Rab3D-MUT reporter (Fig. 6B). Furthermore, we found that transfection with miR-30b-5p mimics significantly suppressed the mRNA and protein expression levels of Rab3D in 786-O and SN12PM6 cells, respectively (Fig. 6C, D).
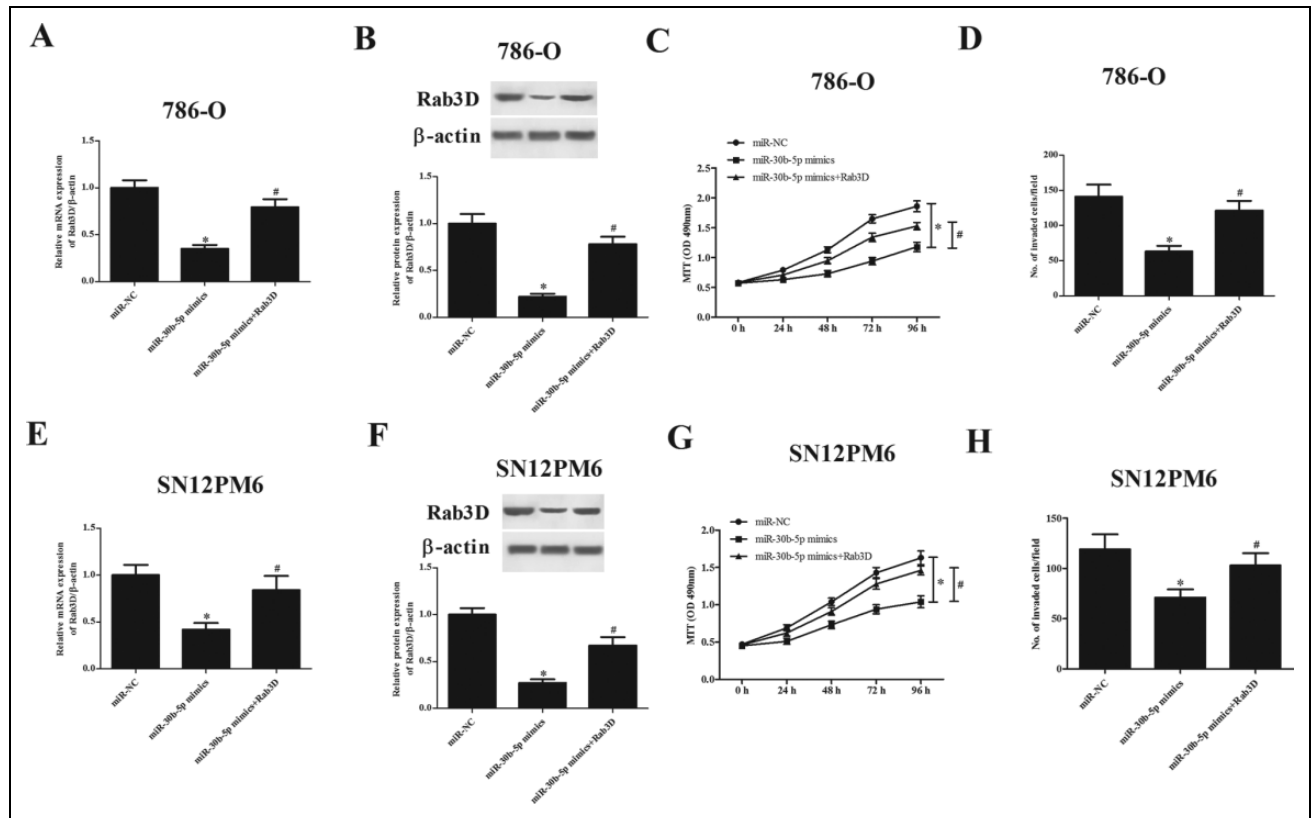
Fig. 7. Overexpression of Rab3D reversed the effects of miR-30b-5p on RCC cell proliferation and invasion. RCC cells were co-transfected with miR-30b-5p mimics and Rab3D-overexpressing vector. (A, B) The expression of Rab3D was measured using qRT-PCR and western blot in 786-O cells. (E, F) The expression of Rab3D was measured using qRT-PCR and western blot in SN12PM6 cells. (C, G) MTT assay was used to detect cell proliferation of 786-O and SN12PM6 cells, respectively. (D, H) Transwell assay was carried out to assess cell invasion in 786-O and SN12PM6 cells, respectively. *$P < 0.05$ versus miR-NC, #$P < 0.05$ versus miR-30b-5p mimics.
miR-NC: miR-30b-5p mimics/negative control; qRT-PCR: qualitative real-time polymerase chain reaction.

Overexpression of Rab3D Reversed the Effects of miR-30b-5p on RCC Cell Proliferation and Invasion

To investigate whether the regulatory effect of miR-30b-5p in RCC cells was due to the direct targeting Rab3D, we detected the effect of Rab3D overexpression on miR-30b-5p-mediated effects. We found that Rab3D overexpression significantly reversed the inhibitory effect of miR-30b-5p on Rab3D expression in 786-O cells (Fig. 7A, B). Similar results were observed in SN12PM6 cells (Fig. 7E, F). Furthermore, we showed that miR-30b-5p mimics transfection resulted in significant decrease in cell proliferation and invasion. However, the effects of miR-30b-5p were mitigated by overexpression of Rab3D in 786-O and SN12PM6 cells, respectively (Fig. 7C, D, G, H).

Knockdown of LINC01133 Inhibited Tumor Growth In Vivo

To explore the impact of LINC01133 on the tumorigenesis of RCC *in vivo*, we established a 786-O cell-derived xenograft model. As shown in Fig. 8A, the weight of 786-O cell-derived tumors was significantly smaller in mice that received LV-sh-LINC01133-infected cells compared with those that received LV-control-infected cells (Fig. 8A). Moreover, tumor volume was also greatly decreased by LINC01133 knockdown (Fig. 8B).

Discussion

LINC01133 has been identified to be implicated in several tumors. Weng et al.[14] demonstrated that LINC01133 is upregulated in pancreatic cancer. Overexpressed LINC01133 *in vitro* exhibits oncogenic effect on pancreatic cancer, as proved by the enhanced growth, proliferation, migration, invasion, and metastasis. Zheng et al.[15] reported that knockdown of LINC01133 inhibits cell proliferation, cell colony formation, migration, invasion, and cell apoptosis, and blocks cell cycle arrest in the G1 phase in hepatocellular carcinoma (HCC) cell lines. *In vivo* tumor xenograft models indicate that knockdown of LINC01133 dramatically represses the tumorigenesis of HCC, which suggests that LINC01133 contributes to the progression of HCC[15]. These findings indicate that LINC01133 acts as an oncogene. However, LINC01133 also exerts antioncogenic role in several cancers[16,17]. For instance, LINC01133 is downregulated in
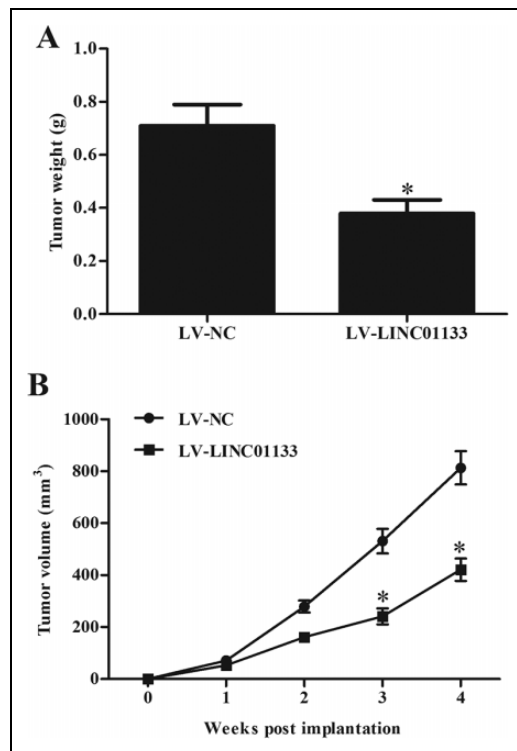
Fig. 8. Effect of LINC01133 knockdown on tumor growth *in vivo*. Xenograft model was established through injection with LV-NC- or LV-LINC01133-infected 786-O cells. (A, B) Tumors were collected for the determination of weight and volume after 4 weeks post injection. *$P < 0.05$ versus LV-NC group.
LV-NC: control lentivirus.

oral squamous cell carcinoma (OSCC)[16]. Moreover, higher expression of LINC01133 is correlated with less metastasis and better prognosis in OSCC patients. *In vitro* experiments prove that LINC01133 inhibits OSCC cell migration and invasion, suggesting that LINC01133 may be a new diagnostic and therapeutic target for OSCC[16]. These dual roles of LINC01133 could attribute to organ-specific actions and different cellular contexts. In the current study, we found that LINC01133 was highly expressed in RCC tissue specimens and cell lines. Downregulation of LINC01133 in RCC cells inhibited RCC cell proliferation, migration, and invasion.

Previous studies have shown that many lncRNAs fulfill their functions by "sponging" target miRNAs and competitively inhibit their biological activities[18,19]. As a novel lncRNA, LINC01133 was identified to sponge miRNAs, thereby achieving its roles in cancers. It has been reported that LINC01133 sponges miR-422a to aggravate the tumorigenesis of human osteosarcoma[12]. In addition, LINC01133 inhibits gastric cancer progression and metastasis by acting as a competitive endogenous RNA for miR-106a-3p[17]. In the present study, we found that miR-30b-5p might be a target of LINC01133. MiR-30b-5p has been demonstrated to act as a tumor suppressor in different cancers. MiR-30b-5p was found to be downregulated in HCC tissues and

correlated with several clinical pathological characteristics, such as pathological stage, tumor size, survival time, differentiation, and intrahepatic metastasis[20]. MiR-30b-5p represses cell proliferation and cell cycle in human HCC cell lines, indicating that miR-30b-5p may be a potential favorable biomarker and therapeutic target for the diagnosis and treatment of HCC[20]. Additionally, expression levels of miR-30b-5p are significantly lower in both RCC tissues and cell lines. Enforced miR-30b-5p expression suppresses cell proliferation, metastasis, and epithelial-to-mesenchymal transition (EMT) in RCC cell lines[21]. Our results proved that LINC01133 directly interacted with miR-30b-5p in RCC cell lines. Knockdown of LINC01133 significantly induced the expressions of miR-30b-5p in RCC cell lines. Moreover, miR-30b-5p exhibited tumor-suppressive activity in RCC, which was evidenced by its inhibitory effects on RCC cell proliferation, migration, and invasion.

Rab3D is a member of the Ras-related GTP-binding protein Rab family and possesses critical biological activities[22,23]. It has been observed that the expression of Rab3D is dysregulated in various types of cancers. Rab3D expression is dramatically increased in colorectal cancer tissues compared to normal tissues[24]. The expression of Rab3D is positively correlated with the tumor size, tumor classification, lymphatic metastasis, distant metastasis, and clinical stage, while negatively related with overall survival in colorectal cancer patients. Besides, increased Rab3D expression is associated with invasiveness of colorectal cancer cells[24]. Rab3D is highly expressed in human esophageal squamous cell carcinoma cells (ESCC) cell lines. In addition, knockdown of Rab3D significantly inhibits the ESCC cell migration, invasion, and EMT, as well as suppresses the tumorigenesis *in vivo*[13]. The current study proved that Rab3D was target gene of miR-30b-5p in RCC cell lines. The inhibitory effects of miR-30b-5p on cell proliferation and invasion were mitigated by overexpression of Rab3D. MiR-30b-5p executed its antitumor property in RCC via targeting Rab3D.

In conclusion, the results demonstrated that LINC01133 acted as an oncogene in RCC. LINC01133 exerted its role through regulation of miR-30b-5p/Rab3D axis in RCC cell lines. Thus, LINC01133 might be a potential therapeutic target for the treatment of RCC.

Ethical Approval

This study was approved by the Ethics Committee at The Second Affiliated Hospital of Medical School, Xi'an Jiaotong University.

Statement of Human and Animal Rights

All procedures in this study were conducted in accordance with The Second Affiliated Hospital of Medical School, Xi'an Jiaotong University of Ethics Committee's (Approval Number: 2020227) approved protocols.

Statement of Informed Consent

Written informed consent was obtained from the patients for their anonymized information to be published in this article.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

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