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RESEARCH PAPER

Linc01133 promotes proliferation and metastasis of human renal cell carcinoma through sponging miR-760

Wei Lv[a], Yan Li[b], Liye Fu[b], Fandong Meng[b], and Jun Li[c]

[a]Department of Cardiology, Shengjing Hospital of China Medical University, Shenyang, China; [b]Department of Biotherapy, Cancer Research Institute, the First Affiliated Hospital, China Medical University, Shenyang, China; [c]Department of Urology, The First Affiliated Hospital, China Medical University, Shenyang, China

ABSTRACT

Renal cell carcinoma (RCC) is one of the most frequent human tumors and has brought great threats to the health of the people around the globe. It was reported that linc01133, a long non-coding RNA (lncRNA), was involved in the pathogenesis and development of several human cancer. But the biological role of linc01133 in RCC is still not understood. The present study aimed to investigate the biological functions of linc01133 in RCC. We did some biological experiments in this study, including quantitative real-time polymerase chain reaction (qRT-PCR), western blotting, MTT assay, wound healing assay, Transwell invasion assay and xenograft tumor assay. In this study, we found the expression levels of linc01133 markedly increased in the RCC tissues compared with the normal tissues. And we found that the over-expressing of linc01133 promoted cell proliferation, migration and invasion, the interfering of linc01133 inhibited cell proliferation, migration and invasion. Furthermore, we found that the interfering of linc01133 inhibited tumor growth in murine xenograft models. Additionally, we found that linc01133 promotes RCC cell proliferation, migration and invasion through sponging miR-760. Collectively, our work preliminarily illuminated the tumor-promoting role of linc01133 in RCC and the potential molecular mechanism. Thus, our study may provide some evidence for the treatment of RCC.

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KEYWORDS
Linc01133; proliferation; invasion; renal cell carcinoma; miR-760

Introduction

Renal cell carcinoma (RCC) is one of the most frequently diagnosed human cancers and has resulted in a considerable amount of deaths around the world [1–4]. It is reported that there are many factors responsible for a large deal of RCC-related deaths, such as high recurrence rate and metastasis potential [5–8]. To improve the life quality and lengthen the lifetime of the people, a great number of attention has been laid to the studies about diagnosis and treatment of RCC. Even through some advances has been obtained, the therapeutic effects and overall survival are still so unsatisfactory. As a consequence, it is a very emergent need to find new and effective therapeutic options.

Long non-coding RNAs (lncRNAs) are a large class of RNA molecules longer than 200 nucleotides, which have no or little protein-coding ability [9–11]. A great number of studies have reported that the change in the expression of lncRNAs is closely linked to the occurrence and development of various human tumors [12–14]. Linc01133 is a cancer-related lncRNA and has been reported to be involved in human lung squamous cell carcinoma [15], non-small cell lung cancer [16] and colorectal cancer [17]. But the biological role of linc01133 in RCC is still not clear. Ras-related protein RAB3D is a member of the Ras oncogene family and has been reported to be over-expressed in several types of human cancers, including human esophageal squamous cell carcinoma [18], lung cancer [19] and colorectal cancer [20].

In this study, our objectives were to investigate the biological role of linc01133 in RCC and find out the possible molecular mechanisms. In this study, we found the expression level of linc01133 in RCC tissues and cell lines notably increased. In addition, we found that the over-expressing of linc01133 promoted proliferation, migration and invasion of RCC cells, the interfering of linc01133

CONTACT Jun Li Lijun_7021@163.com Department of Urology, The First Affiliated Hospital, China Medical University, 155 North Nanjing Street, Heping District, Shenyang110001, Liaoning Province, China

inhibited proliferation, migration and invasion. Moreover, we found that linc01133 executed its roles through positive regulating RAB3D by miR-760.

Materials and methods

Tissue sample collection

The present study was approved by the Ethical Committee of First Affiliated Hospital of China Medical University [approval code: (2021) 490]. Written informed consent was obtained from all subjects. Tumor tissues and matched non-cancerous tissues were obtained from 70 patients with RCC who underwent surgical resection at the First Affiliated Hospital of China Medical University (Shenyang, China). All the patients did not received radiotherapy and immunotherapy.

Cell culture

Human kidney cell line HKC and four human RCC cell lines (786-O, Caki-1, ACHN and OS-RC-2) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All the cells were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT, USA) containing 10% fetal bovine serum (FBS; Sangon Biotechnology, Shanghai, China). All the cells were maintained at 37°C in a humidified incubator containing 5% CO_2. Only cells in logarithmic growth phase were used throughout this study.

Cell transfection

In brief, cells were plated in 6-well plates at a density of 1×10^5 cells/well and incubated at 37°C until they were about 80% confluent before transfection. The oligonucleotides of miR-760 mimics, miR-760 inhibitor and their negative controls (NC mimics and NC inhibitor) were purchased from GenePharma (Shanghai, China). A pBABE-puro-LINC01133 expression vector and RNA interference lentiviral pLKO.1 plasmid vector targeting LINC01133 were also constructed by GenePharma. Cell transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

MTT assay

Cell proliferation was evaluated by MTT assays. Briefly, cells were seeded at 96-well plates at a density of 5×10^3 cells/ well. The MTT regent (Shanghai Sangon, Shanghai, China) was added into each well at different time points and incubated at 37°C for 4 h. The OD value was read at 570 nm using a microscope spectrophotometer (Thermo Fisher Technologies, USA).

Wound healing assays

Wound healing assays were performed to examine the migration ability of cells. A scratch was formed on the mono-layer of cells using a sterile plastic tips. After 24 h of incubation, the closure of the scratch was observed. Images of cells migrating into the wound were captured at 0 and 24 h using an inverted microscope (CKX31, Olympus) at 40× magnification. In each field, the distance from the margin of the lesion to the 10 most migrated cells were measured, and the mean value of the distances was taken as the mobility of cells in each culture dish. All the experiments were carried out in triplicates.

Transwell invasion assays

Transwell assays were conduct to detect the invasion capabilities of cells. Cells (4×10^5) were plated within the top chamber coated with Matrigel membrane. 10% FBS in the lower chamber was used as a chemoattractant. After 48 h incubation, the noninvading cells remaining on the upper surface of the membranes were removed by a cotton swab. Cells on the lower surface of the membranes were fixed with 4% formaldehyde for 10 min at room temperature and stained with 0.5% crystal violet for 10 min. Five fields of adherent cells were counted randomly in each well under an inverted microscope (Olympics) at magnification of 200 × . All the experiments were performed in triplicates.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNAs were extracted from tissues or cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed to complementary (cDNA) using a Reverse Transcription Kit (Thermo Fisher, Waltham, MA, USA). Reverse transcription reactions were carried out using 100 ng RNA, 50 nM/l stem-loop RT primers, 1X RT buffer, 0.25 mM/l of each dNTP, 3.33 U/μl MultiScribe reverse transcriptase and 0.25 U/μl RNase inhibitor. The quantity and quality of total RNA and cDNA were determined by spectrophotometer and agarose gel electrophoresis, respectively. Quantitative PCR analysis was conducted using a standard protocol from SYBR Green qPCR Mix (Takara, Dalian, China). Next, qRT-PCR was performed using a 7500 Fast Real-Time PCR System (Applied Biosystems, Waltham, MA, USA), with an initial denaturation for 10 min at 95°C, primer annealing at 50°C for 2 min, followed by 40 cycles of 15 seconds at 95°C and 1 min at 60°C. The primers used in this study was synthesized by Shanghai Sangon Biotechnology, which showed as follows: LINC01133, 5'-TGGGAAAGAGGTTGCAGT-3' (F) and 5'-CCAAAGGGAAGCTAAGGAG-3' (R); miR-760, 5'-CGGCTCTGGGTCTGTGGGGA-3' (F) and R: 5'-CTCTACAGCTATATTGCCAGCCA-3' (R); RAB3D, 5'-GACCTCCGGTTTAGAGGCAC-3' (F) and R: 5'-GTTGGTTGGTGTGTGGGAGC-3' (R); GAPDH, 5'-GGGAGCCAAAAGGGTCAT-3' (F) and 5'-GAGTCCTTCCACGATACCAA-3' (R); U6, 5'-CTCGCTTCGGCAGCACA-3' (F) and 5'-AACGCTTCACGAATTTGCGT-3' (R). The relative expression level was calculated using $2^{-\Delta\Delta Ct}$ method with GAPDH or U6 as the negative control. All the experiments were conducted in triplicates.

Luciferase reporter assay

Wild-type RAB3D 3'UTR fragment and mutant RAB3D 3'UTR fragments were inserted into pmirGLO reporter vectors (Promega), respectively. Cells were co-transfected with miR-760 mimics and wild-type RAB3D 3'UTR or mutant RAB3D 3'UTR fragments by Lipofectamine 2000 (Invitrogen). Relative luciferase activity was measured on a dual-luciferase reporter assay system (Promega) at 48 h post-transfection. Similar experiments were carried out to validate the binding of miR-760 and linc01133. Data were expressed as the ratio of Renilla luciferase activity to firefty luciferase activity.

In situ hybridization (ISH)

Tissue slides were pre-hybridized in a hybridization solution (Boster Bioengineering Co., Ltd, Wuhan, China) at 37°C for 2 hours. Then digoxigenin-labeled detection probes (Boster) were added and hybridized overnight at 37°C. After three washes, an immunologic reaction was performed with a mouse monoclonal antibody to digoxigenin, followed by supplementing alkaline phosphatase-coupled streptavidin dilution (BD Bioscience) to detect streptavidin dilution probes. Slides were mounted with aqueous mounting medium (Maixin Biotechnology). All the experiments were performed in triplicates.

RNA-binding protein immunoprecipitation (RIP)

RIP assays were carried out using EZ-Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore) following the manufacturer's instructions. Briefly, RIP buffer containing magnetic beads coated coupled with human anti-Ago2 antibody (Millipore) and negative control IgG was added to cell lysate, respectively. Cell lysates were then incubated overnight at 4°C. We then digest the proteins using Proteinase K and isolated the co-precipitated RNAs. Finally, PCR analysis was performed to determine the purified RNAs. All the experiments were conducted in triplicates.

Western blotting assay

Cells were lysed uisng RIPA buffer (Sangon) containing a protease inhibitor cocktail and PMSF. The concentrations of total proteins were determined by BCA Kit (TaKaRa, Dalian, China). Equal amounts of protein (30 μg) were separated via 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and subsequently transferred onto transferred on PVDF membranes (Takara). The membranes were blocked using 5% nonfat dry

milk solution in TBS buffer containing 0.1% Tween 20 at 37°C for 30 min. The membranes were then incubated with diluted primary antibodies against Rab3D (ab133301; 1/1000 dilution) and β-actin (ab115777; 1/200 dilution) (all from Abcam, Shanghai, China) overnight at 4°C. After three washes, the membranes were then incubated with goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibodies (ab205718) at 1/50,000 dilution at 37°C for 1 h. An ECL Detection Kit (Takara) was used to determine the blotting. All the experiments were performed in triplicates.

Tumor transplantation of renal cell carcinoma in nude mice

A total of 10 BALB/c nude mice (5–6 week of age) were purchased from Shanghai Laboratory Animal Center (Shanghai, China). Cells transfected with shRNA-NC or shRNA-linc01133 (2×10^6 cells per mouse) were subcutaneously administrated into the flanks of the nude mice, respectively. The length and width of tumors were measured using a caliper every 3 days. After 21 days, the tumor nodules of the mice were then removed and weighed. The tumor volume was calculated using modified ellipse volume formula: tumor volume (mm^3) = length (mm)×width $(mm)^2/2$.

Immunohistochemistry (IHC) assay

Samples were fixed in 10% neutral buffered formalin, embedded in paraffin, and sliced into thin sections (5 μm). After being dewaxed and rehydrated, sections were incubated with 3% H_2O_2 for 30 min to block the endogenous peroxidase (POD) activity. Following antigen retrieval (AR) by microwave (heating), 5% bovine serum albumin (BSA) was applied to block nonspecific binding. The sections were then incubated with primary antibodies at 4°C overnight. After being rinsed with PBS three times for 5 min each, sections were treated with biotinylated secondary antibody (Abcam) for 1 h, followed by incubation with streptavidin-horseradish peroxidase (HRP) for 20 min. Diaminobenzidine (DAB) substrate was used as color developing agent. All the experiments were carried out in triplicates.

Statistical analysis

Results were presented as mean ± standard deviation (SD). SPSS 17.0 software was used to perform statistical analysis. The Kolmogorov-Smirnov test was used for the confirmation of data normality. Differences between two groups were analyzed using two-tailed Student's *t*-test. One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was applied to analyze the difference among three or more groups. Pearson's correlation analysis was conducted to determine the relationship between linc01133 expression and miR-760 or RAB3D mRNA expression in the tumorous tissues. $P < 0.05$ was considered statistically significant.

Results

The expression of linc01133 was significantly increased in RCC tissues

It was reported that linc01133 was involved in the pathogenesis and development of several human neoplasms, but its role in RCC is still not known. In this study, we adopted qRT-PCR assays to examine the expression levels of linc01133 in 70 paired RCC tissues and normal tissues. The results of qRT-PCR assays showed that the expression levels of linc01133 in RCC tissues were significantly increased compared with the normal tissues (Figure 1a; $P < 0.0001$). The results of ISH assays also showed that the expression of linc01133 was markedly increased in the RCC tissues compared with the normal tissues (Figure 1b). And the results of qRT-PCR assays showed that the expression of linc01133 in RCC cell lines was significantly up-regulated compared with normal human kidney cell line HKC (Figure 1c; $^{**}P < 0.01$, $^{***}P < 0.001$). The results mean that the expression of linc01133 is markedly increased in RCC.

Linc01133 promoted cell proliferation, migration and invasion

To understand the role of linc01133 in RCC, we did further investigations. The results of MTT assays showed that the over-expressing of linc01133 promoted cell proliferation compared

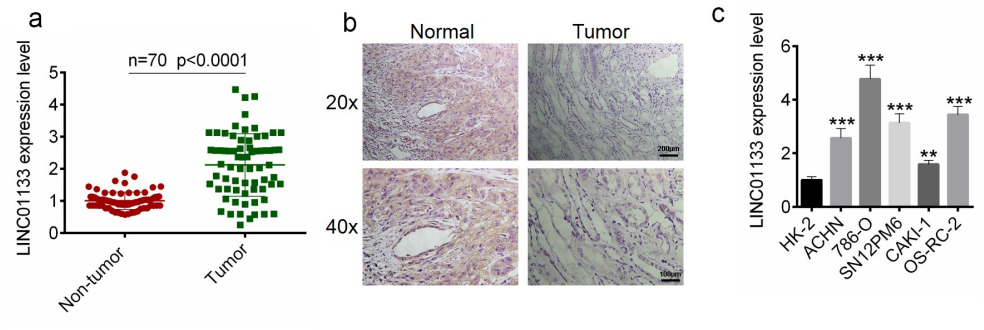

Figure 1. The expression of linc01133 was markedly increased in RCC tissues. A. Linc01133 expression levels in 70 pairs of RCC tissues and normal tissues were determined by qRT-PCR analysis (P < 0.0001). B. Linc01133 expression profiling in the RCC tissues and normal tissues of selected 5 patients was analyzed by ISH. C. Linc01133 expression levels in normal human kidney cell line HKC and four RCC cell lines were analyzed by qRT-PCR. ***P* < 0.01; ****P* < 0.001.

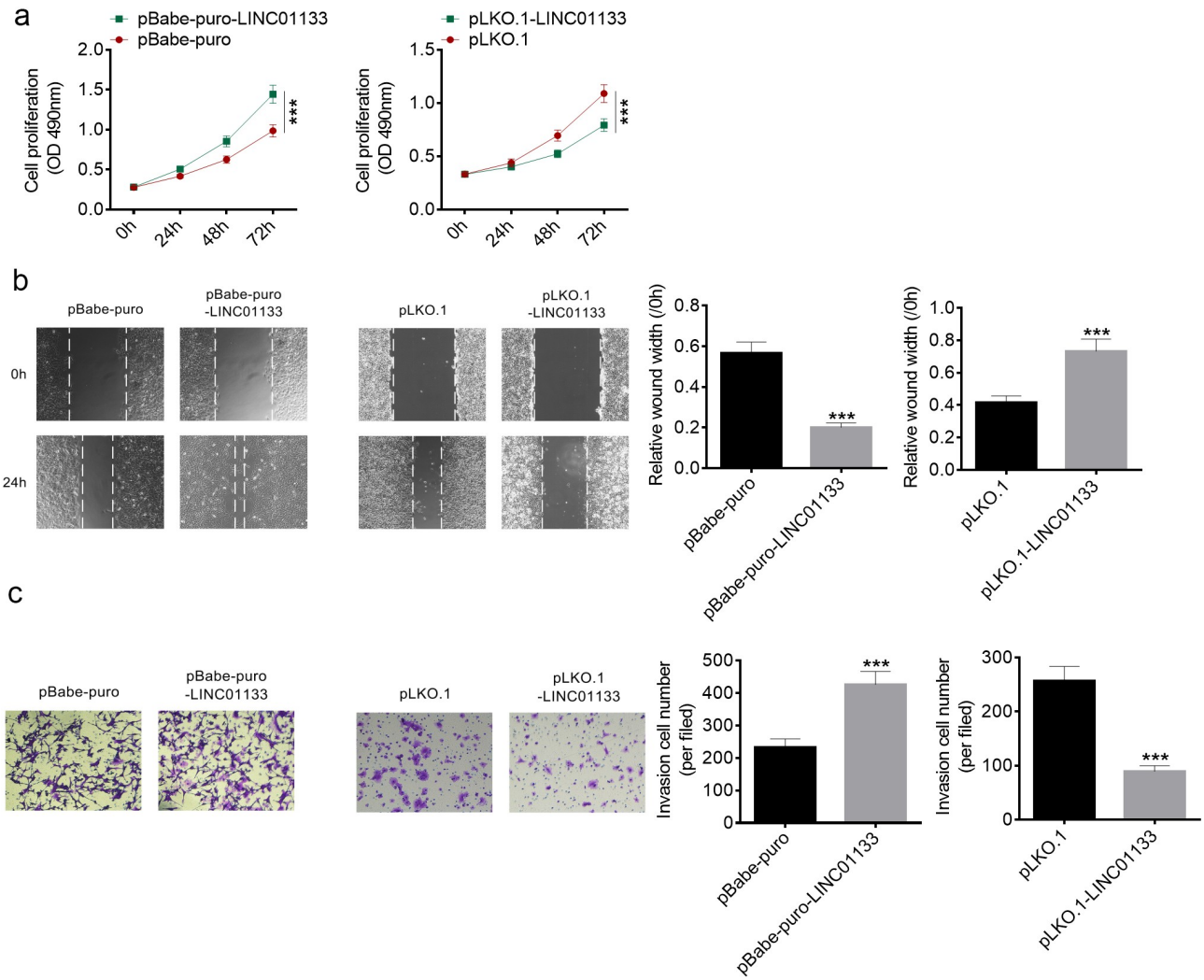

Figure 2. Linc01133 promoted cell proliferation, migration and invasion. A. Cell proliferation was analyzed by MTT assays after the over-expressing and interfering of linc01133 in CAK-1 and 786-O cells, respectively. B. Cell migration was analyzed by wound healing assays after the over-expressing and interfering of linc01133 in CAKI-1 and 786-O cells, respectively. C. Cell invasion was analyzed by Transwell invasion assays after the over-expressing and interfering of linc01133 in CAKI-1 and 786-O cells, respectively. ****P* < 0.001.

with control group, the interfering of linc01133 inhibited cell proliferation (Figure 2a; ***$P < 0.001$). As shown in Figure 2b, the over-expressing of linc01133 promoted cell migration compare with control group, the interfering of linc01133 inhibited cell migration (***$P < 0.001$). The results of Transwell invasion assays showed that the over-expressing promoted cell invasion compared with the control group, the interfering of linc01133 inhibited cell invasion (Figure 2c; ***$P < 0.001$). The results mean that linc01133 promotes cell proliferation, migration and invasion.

Linc01133 binds to miR-760 in RCC cells

We used the miRanda algorithms to predict the miRNAs which possibly bound to linc01133. As shown in Figure 3a, miR-760 was selected as a candidate target of linc01133. The results of RIP assays showed that more miR-760 was enriched by the over-expressing of linc01133 compared with the negative control group (Figure 3b; ***$P < 0.001$). The results of qRT-PCR assays showed that the over-expressing of linc01133 dramatically decreased the expression of miR-760 compared with the negative control group, the interfering of linc01133 increased the expression of miR-760 (Figure 3c; ***$P < 0.001$). The results mean that linc01133 binds to miR-760 in RCC cells.

Linc01133 regulated the expression of RAB3D by sponging miR-760

We used the miRanda software to predict the possible target mRNAs of miR-760. RAB3D, which was reported to be involved in several human cancers, was chosen as a candidate target (Figure 4a). The results of luciferase reporter assays showed that the co-transfection of miR-760 and wild-type RAB3D 3'UTR significantly decreased the activities of luciferase, the co-transfection of miR-760 and mutant RAB3D 3'UTR had no significant effects on the activities of luciferase (Figure 4b; ***$P < 0.001$). As shown in Figure 4c, the transfection of miR-760 mimics decreased the expression of RAB3D, the transfection of miR-760 inhibitor increased the expression of RAB3D (***$P < 0.001$). As shown in Figure 4d, the over-expressing of linc01133 increased the expression of RAB3D, the interfering of linc01133 decreased the expression of RAB3D (***$P < 0.001$). And we found that linc01133 expression was negatively related to the expression of miR-760 and that the expression of linc01133 was positively related to the mRNA expression of

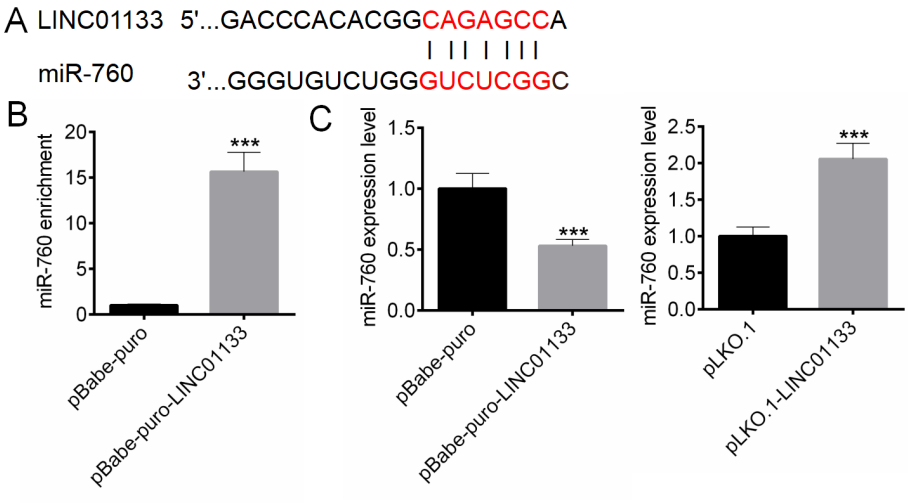

Figure 3. Linc01133 binds to miR-760 in RCC cells. A. A binding site of linc01133 in miR-760 was forecasted by miRanda online tools. B. RIP assays were carried out to enrich miR-760, followed by qRT-PCR analysis. C The expression levels of miR-760 was determined by qRT-PCR assays after the over-expressing and interfering of linc01133 in CAKI-1 and 786-O cells, respectively. ***$P < 0.001$.

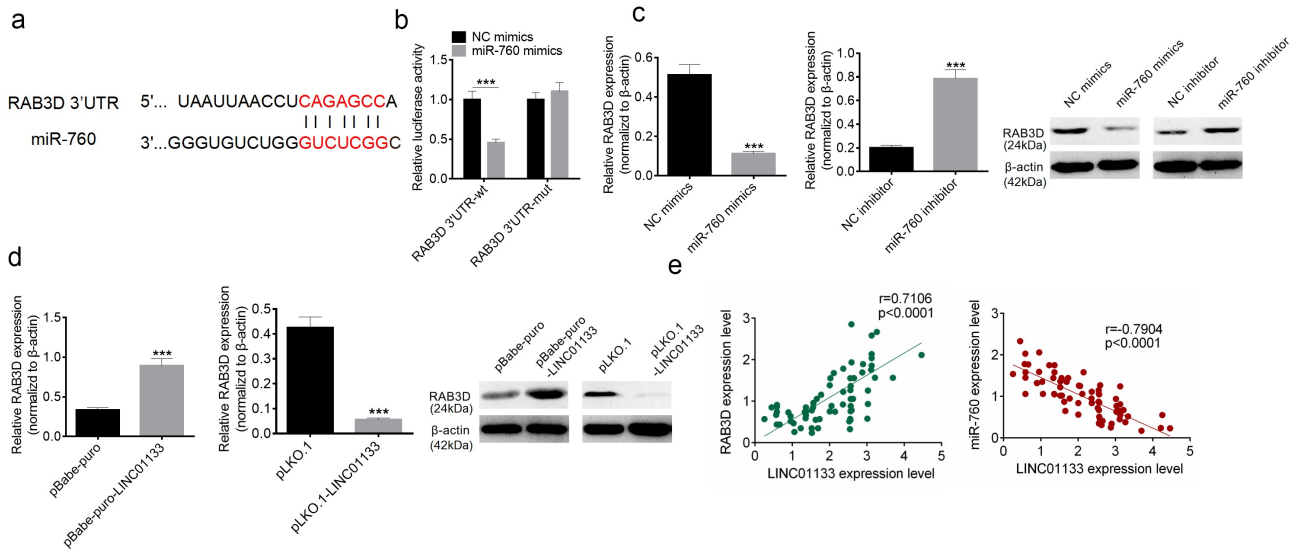

Figure 4. Linc01133 regulated RAB3D expression by sponging miR-760. A. A binding site of miR-760 in RAB3D 3'UTR was forecasted by TargetScan online tools. B. Luciferase activity was analyzed after co-transfection of miR-760 and wild-type or mutant RAB3D 3'UTR fragments. C. RAB3D protein expression levels were analyzed after transfection with miR-760 mimics or miR-760 inhibitor. D. RAB3D protein expression levels were analyzed after the over-expressing and interfering of linc01133 in CAKI-1 and 786-O cells, respectively. ***$P < 0.001$. E. The relationship between linc01133 expression and miR-760 or RAB3D mRNA expression in the RCC tissues ($P < 0.0001$).

RAB3D (Figure 4e; $P < 0.0001$). The results mean that linc01133 regulates the expression of RAB3D by sponging miR-760.

Linc01133 promoted cell proliferation, migration and invasion by sponging miR-760

To further investigate the possible molecular mechanism, we carried out the following studies. As shown in Figure 5a-**C**, miR-760 inhibitor decreased the suppressive effects of the interfering of linc01133 on cell proliferation, migration and invasion (**$P < 0.01$, ***$P < 0.001$). In addition, we found that miR-760 inhibitor markedly increased expression the levels of RAB3D in the RCC cells treated with sh-linc01133 (Figure 5d; ***$P < 0.001$). The results mean that linc01133 promotes cell proliferation, migration and invasion by sponging miR-760.

The interfering of linc01133 inhibited tumor growth in murine transplantation models

To analyze the effects of linc01133 on tumor growth, we built nude mice tumor-bearing models. As shown in Figure 6a, the interfering of linc01133 inhibited tumor growth in murine xenograft models (***$P < 0.001$). The results of IHC assays showed that the interfering of linc01133 decreased Ki67 and MMP9 expression in the tumors excised from the nude mice compared with the control group (Figure 6b). The results mean that the interfering of linc01133 inhibited tumor growth in murine transplantation models.

Discussion

RCC is one of the most common and lethal human neoplasms, which has already brought great threat to the people worldwide [21,22]. Along with the development in the medicine field, more and more methods to diagnose and treat RCC have already been sought. Although the significant progress has been gained in the diagnosis and treatment of human tumors, the long-term prognosis and the effects of therapy are still unsatisfactory. The past studies have reported that the abnormal expression of lncRNAs plays important roles in the pathogenesis and development of human cancers [23–25]. Jing *et al* [15] reported that linc01133 was up-regulated in lung squamous cell cancer and predicted survival. Zang *et al* [16] reported that

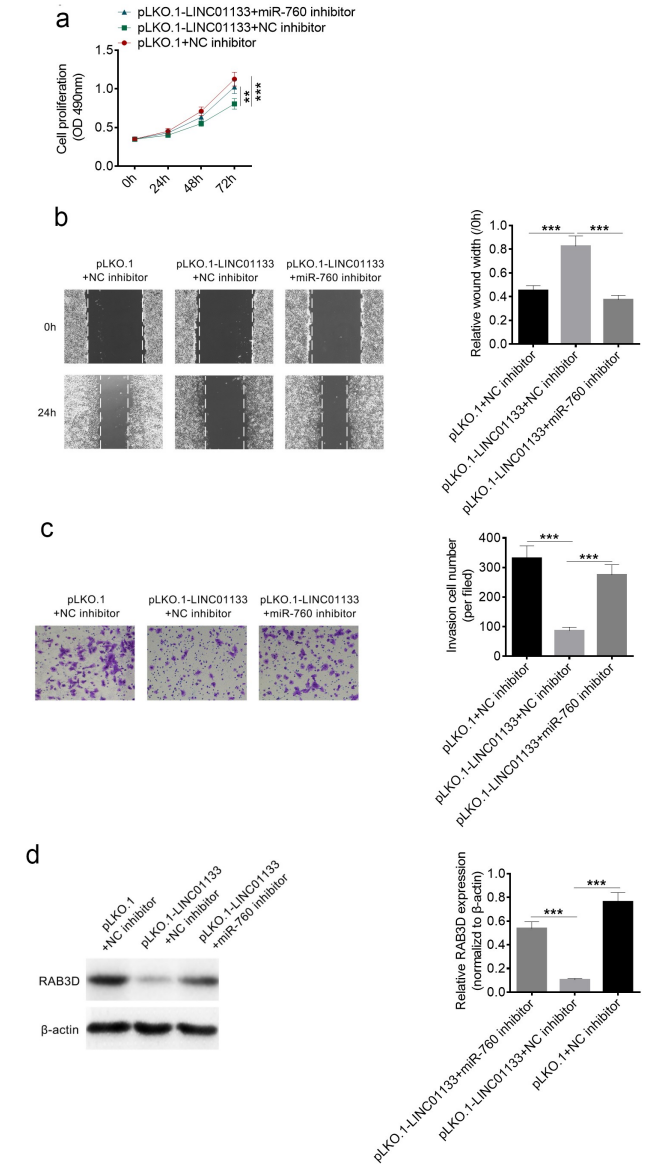

Figure 5. Linc01133 promoted cell proliferation, migration and invasion by sponging miR-760. A. Cell proliferation after co-transfection of shRNA-linc01133 and miR-760 inhibitor in the 786-O cells. B. Cell migration after co-transfection of shRNA-linc01133 and miR-760 inhibitor in the 786-O cells. C. Cell invasion after co-transfection of shRNA-linc01133 and miR-760 inhibitor in the 786-O cells. D. RAB3D protein expression levels after co-transfection of shRNA-linc01133 and miR-760 inhibitor in the 786-O cells. $^{**}P < 0.01$; $^{***}P < 0.001$.

linc01133 was up-regulated in non small cell lung cancer. Zhang et al [17] reported that linc01133 was down-regulated in colorectal cancer tissues and was an independent prognostic factor. But the role of linc01133 in RCC is still not clear. As a consequence, to investigate the role of linc01133 in RCC may be beneficial to better catching on the potential mechanisms of the occurrence and progression of human RCC.

In this study, we did qRT-PCR and ISH analysis to examine the expression levels of linc01133 in the RCC tissues and normal tissues, and found the expression levels of linc01133 dramatically increased in the RCC tissues compared with the normal tissues. Therefore, we guessed that linc01133 was involved in the pathogenesis and development of RCC. In the further researches, we found that the over-expressing of linc01133 promoted RCC cell proliferation, migration and invasion, but the interfering of linc01133 inhibited RCC cell proliferation, migration and invasion. In the following researches, we found that linc01133 positively regulated RAB3D

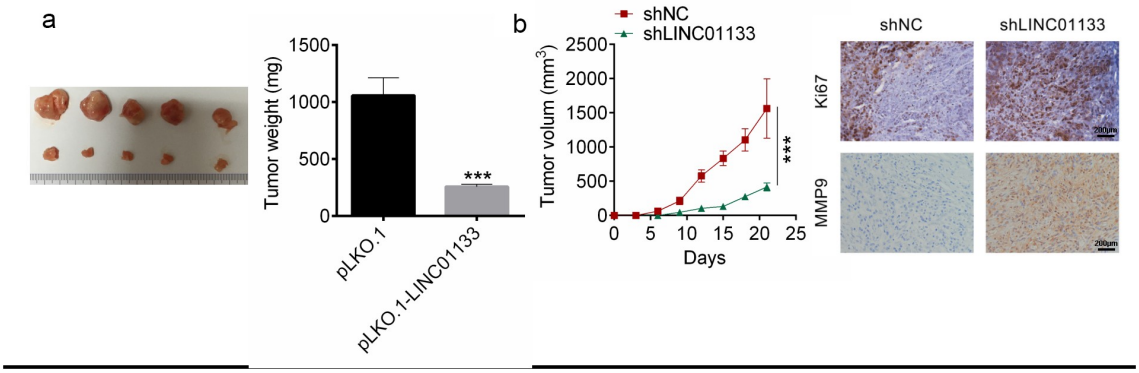

Figure 6. The interfering of linc01133 inhibited tumor growth in murine transplantation models. A. 786-O cells treated with shRNA-linc01133 were injected into the flanks of the nude mice. Tumor volume was measured every three days. Three weeks later, the nude mice were sacrificed and tumors were weighed. B. Ki67 and MMP9 protein expression in the tumors was analyzed by IHC. ***$P < 0.001$.

to promote cell proliferation, migration and invasion by sponging miR-760.

An increasing number of researchers agree to the proposal that lncRNAs may serve as miRNA sponges. miRNA-760 has been reported to suppress carcinogenesis and tumor progression in many human tumors. Han et al [26] reported that miR-760 suppressed cancer stem cell subpopulation, proliferation and metastasis in breast cancer. Cao et al [27] reported that miR-760 inhibited human colorectal cancer growth by targeting BATF3/AP-1/cyclinD1 signaling. Yan et al [28] reported that miR-760 inhibited cell proliferation and metastasis by targeting ROS1 in non small cell lung cancer. RAB3D is known as a member of the Rab oncogene family and contributes to the occurrence and development of several human cancers [18–20]. Our study indicated that RAB3D expression was regulated by linc01133/miR-760 axis in RCC. Nevertheless, one limitation in this study was that we were unable to perform RNA sequencing to explore the lncRNA expression profiles in RCC tissues.

In sum, we found the expression of linc01133 was markedly increased in the RCC tissues compared with matched normal tissues and that the expression of linc01133 was also up-regulated in RCC cells. Moreover, our study showed that linc01133 promoted RCC cell proliferation, migration and invasion through miR-760/RAB3D pathway. Our results suggest that linc01133 may be used as a potential therapeutic target for RCC patients.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Availability of data and materials

All data generated and/or analyzed during this study are included in this published article.

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