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Long noncoding RNA ITGB1 promotes migration and invasion of clear cell renal cell carcinoma by downregulating Mcl-1

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Abstract. – OBJECTIVE: Researchers have discovered the important role of long noncoding RNA (lncRNAs) in tumorigenesis recently. In this work, we aimed to explore whether lncRNA linc-ITGB1 affected the development of clear cell renal cell carcinoma (ccRCC), and to elucidate the possible underlying mechanism.

PATIENTS AND METHODS: Linc-ITGB1 expression in both ccRCC cells and tissue samples was detected by Real Time-quantitative Polymerase Chain Reaction (RT-qPCR). Moreover, the association between linc-ITGB1 expression level and patients' disease-free survival rate was explored. Then, wound healing and transwell assays were conducted. Furthermore, the underlying mechanism was explored through RT-qPCR and Western blot assay.

RESULTS: Linc-ITGB1 expression level in ccRCC samples was markedly higher than that of the adjacent ones. The expression of linc-ITGB1 was closely related to the disease-free survival time of ccRCC patients. Moreover, the migration and invasion of ccRCC cells were remarkably enhanced after linc-ITGB1 upregulation *in vitro*. In addition, the mRNA and protein expression of Mcl-1 were significantly downregulated after linc-ITGB1 overexpression. Furthermore, the expression level of Mcl-1 was negatively correlated with the linc-ITGB1 expression in ccRCC tissues.

CONCLUSIONS: Our findings suggested that linc-ITGB1 could enhance ccRCC cell migration and invasion via downregulating Mcl-1. In addition, linc-ITGB1 might be a potential therapeutic target for ccRCC.

Key Words
Long noncoding RNA, Linc-ITGB1, Clear cell renal cell carcinoma (ccRCC), Mcl-1.

Introduction

Renal cell carcinoma (RCC) is the most common kidney carcinoma, ranking the sixth leading cause of cancer-related death in America. Males are more likely to be diagnosed with RCC, with a ratio of 1.6:1.0 between males and females. Most patients are diagnosed at the age between 40 to 70 years old[1]. As the major subtype of renal epithelial malignancies, clear cell renal cell carcinoma (ccRCC) accounts for about 70%-80% of all RCC diagnoses[2]. Most ccRCC patients can be cured by surgical excision. However, due to local or distant metastasis, 30% of newly diagnosed ccRCC cases present a median survival time of 13 months[3,4]. Therefore, it is urgent to elucidate the underlying mechanism and find new therapeutic strategies.

Recent researches have revealed that lncRNA ITGB1 functions as a novel oncogene in tumorigenesis[5-7]. However, the exact role of linc-ITGB1 in ccRCC remains unknown. In this study, we found that the expression of linc-ITGB1 was remarkably higher in ccRCC tissues. Moreover, linc-ITGB1 could remarkably promote the migration and invasion of ccRCC cells *in vitro*. Moreover, we further explored the underlying mechanism of linc-ITGB1 function in the ccRCC development.

Patients and Methods

Cell Lines and Clinical Samples

60 ccRCC patients who received surgery at the Affiliated Yantai Yuhuangding Hospital of

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Qingdao University were enrolled in this study. Meanwhile, human tissues were collected. No radiotherapy or chemotherapy was performed before the surgery. Samples obtained from the surgery were immediately preserved at -80°C. All tissues were confirmed by an experienced pathologist. This investigation was approved by the Research Ethics Committee of the Affiliated Yantai Yuhuangding Hospital of Qingdao University. Informed consent was obtained from each patient before the study.

Cell Culture

Human renal cancer cell lines (Caki-1, 769-P, 786-O, ACHN) and human kidney epithelial cell (HK-2) were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Science (Shanghai, China). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and penicillin, and maintained in a 5% CO_2, 37°C incubator.

Cell Transfection

Lentiviral virus targeting linc-ITGB1 was synthesized and cloned into a pLenti-EF1a-EGFP-F2A-Puro vector (Biosettia Inc., San Diego, CA, USA). Linc-ITGB1 lentiviruses (linc-ITGB1) and empty vector (control) were packaged in 293T cells. Subsequently, they were transfected into ccRCC cells. 48 h later, Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) was used to detect the linc-ITGB1 expression level in the transfected cells.

RNA Extraction and RT-qPCR

Total RNA was extracted according to the instructions of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Extracted total RNA was reverse transcribed into complementary deoxyribose nucleic acid (cDNA) through the reverse Transcription Kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). The thermal cycle was as follows: 30 sec at 95°C, 5 sec for 40 cycles at 95°C, 35 sec at 60°C. Primer sequences used in this study were as follows: Linc-ITGB1, F: 5'-AACCAGGCCCCTCCTTACTC-3', R: 5'-GATGTGTCCGAAGGCTAGGA-3'; Mcl-1, F: 5'-GTGTAGGGAACATCCTCGACTG-3', R: 5'-CGTGTATTGCGTGGAGTCG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Western Blot Analysis

Total protein was extracted from cells by radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China). The concentration of extracted protein was detected by the BCA protein assay kit (TaKaRa, Dalian, China). Subsequently, the protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were then incubated with the corresponding primary and secondary antibodies. Rabbit anti-GAPDH and rabbit anti-Mcl-1 were provided by Cell Signaling Technology (CST; Danvers, MA, USA), as well as goat anti-rabbit secondary antibody. Image J software was applied for the assessment of protein expression.

Wound Healing Assay

After seeded into 6-well plates, the cells were cultured in DMEM medium overnight. Then, the cells were scratched with a 200 μL plastic tip, followed by culture in serum-free DMEM. Each assay was repeated in triplicate independently. The wound closure was observed at different time points.

Transwell Assay

5×10^4 cells in 200 μL serum-free DMEM were added to the upper chamber of an 8 μm pore size insert (Millipore, Billerica, MA, USA). Meanwhile, DMEM and FBS were added to the lower chamber. 48 h later, the top surface of chamber was immersed in pre-cooled methanol for 10 min after being wiped by a cotton swab. Then the cells were stained with crystal violet for 30 min. Three fields were randomly selected for each sample, and the number of migrating cells was counted.

Matrigel Assay

5×10^4 cells in 200 μL serum-free DMEM were added to the upper chamber of an 8 μm pore size insert (Millipore, Billerica, MA, USA) coated with 50 μg Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Meanwhile, DMEM and FBS was added to the lower chamber. 48 h later, the top surface of the chamber was immersed in pre-cooled methanol for 10 min after being wiped by a cotton swab. Then, the cells were stained with crystal violet for 30 min. Three fields were ran-

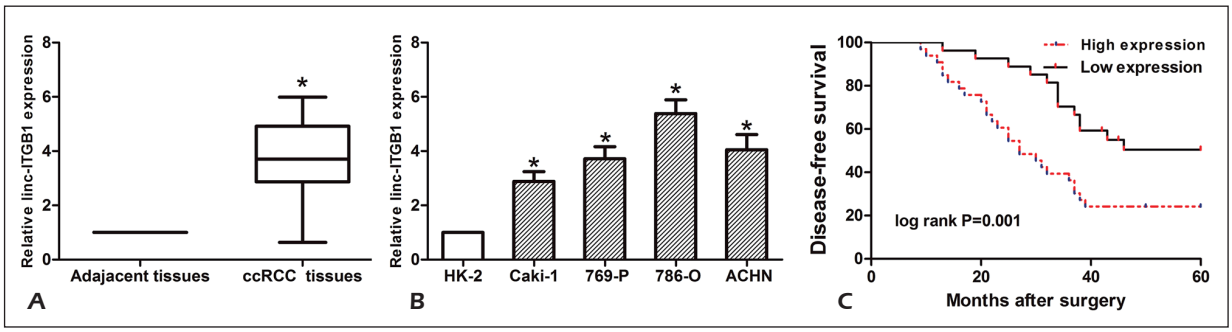

Figure 1. Expression of linc-ITGB1 in ccRCC tissues and cell lines. **A**, Linc-ITGB1 expression in ccRCC tissues was significantly increased when compared with adjacent tissues. **B**, The expression levels of linc-ITGB1 relative to β-actin in human ccRCC cell lines and 16HBE (normal human bronchial epithelial cell) were determined by RT-qPCR. **C**, Higher level of linc-ITGB1 was associated with worse disease-free survival of ccRCC patients. Data were presented as mean ± standard error of mean. *p<0.05.

domly selected for each sample, and the number of invading cells was counted.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 (SPSS, Chicago, IL, USA) was used for all statistical analysis. Data were presented as mean ± SD. Student *t*-test and Kaplan-Meier method were performed for analysis. p<0.05 was considered statistically significant.

Results

Linc-ITGB1 Expression Level in ccRCC Tissues and Cells

The linc-ITGB1 expression in 60 tissue samples and 4 ccRCC cell lines was detected *via* RT-qPCR. As a result, linc-ITGB1 was significantly upregulated in ccRCC tissue samples (Figure 1A). Meanwhile, linc-ITGB1 expression level in ccRCC cells was remarkably higher than that of HK-2 cells (human kidney epithelial cell) (Figure 1B).

Higher Expression of Linc-ITGB1 Was Correlated With Worse Disease-Free Survival of ccRCC Patients

The survival rate of ccRCC patients after surgery was analyzed by the Kaplan-Meier method. According to the median expression, 60 ccRCC patients were divided into two groups: the high-linc-ITGB1 group and the low-linc-ITGB1 group. The Kaplan-Meier analysis showed that the disease-free survival of ccRCC patients in the high-linc-ITGB group was significantly worse than that of the low-linc-ITGB1group (Figure 1C).

Overexpression of Linc-ITGB1 Promoted Migration and Invasion of ccRCC Cells

In this study, Caki-1 and 786-O ccRCC cell lines were chosen for linc-ITGB1 overexpression. Then, the linc-ITGB1 expression in the transfected cells was confirmed by qRT-PCR (Figure 2A). Moreover, wound healing assay results indicated that the overexpression of linc-ITGB1 significantly enhanced the migration ability of ccRCC cells (Figure 2B). Furthermore, transwell assay revealed that the number of migrated and invaded ccRCC cells was remarkably increased after linc-ITGB1 overexpression (Figure 3A, 3B).

Interaction Between Mcl-1 and Linc-ITGB1 in ccRCC

RT-qPCR results showed that the expression level of Mcl-1 in ccRCC cells of the linc-ITGB1 lentiviruses (linc-ITGB1) group was significantly lower than that of the empty vector (control) group (Figure 4A). The results of the Western blot assay demonstrated that after linc-ITGB1 overexpression, the protein expression of Mcl-1 was remarkably downregulated (Figure 4B). Furthermore, we found that Mcl-1 expression in ccRCC tissues was significantly lower when compared with adjacent tissues (Figure 4C). The correlation analysis demonstrated that the Mcl-1 expression level was negatively correlated with the linc-ITGB1 expression in ccRCC tissues (Figure 4D).

Discussion

LncRNAs are a type of newly discovered subgroups of noncoding RNAs (ncRNAs), which

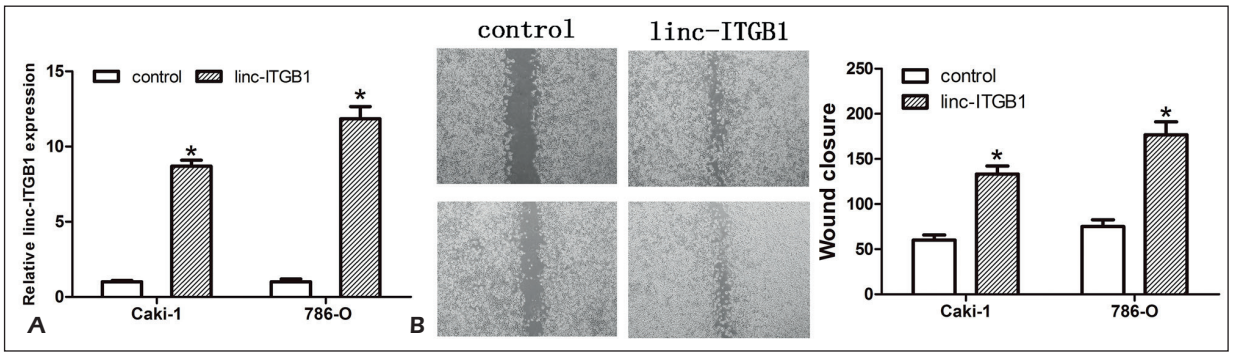

Figure 2. Overexpression of linc-ITGB1 promoted ccRCC cell migration. **A**, Linc-ITGB1 expression in ccRCC cells transfected with linc-ITGB1 lentiviruses (linc-ITGB1) and empty vector (control) was detected by RT-qPCR. β-actin was used as an internal control. **B**, Wound healing assay showed that the overexpression of linc-ITGB1 significantly increased migration of ccRCC cells (Magnification: 10×). The results represented the average of three independent experiments (mean ± standard error of the mean). *p<0.05, as compared with control cells. *p<0.05.

are longer than 200 nucleotides. LncRNAs cannot be translated into proteins. However, studies have revealed that lncRNAs play a crucial role in a variety of biological processes, including proliferation, migration and metastasis of cancer. For example, lncRNA HORAIRM1 inhibits the

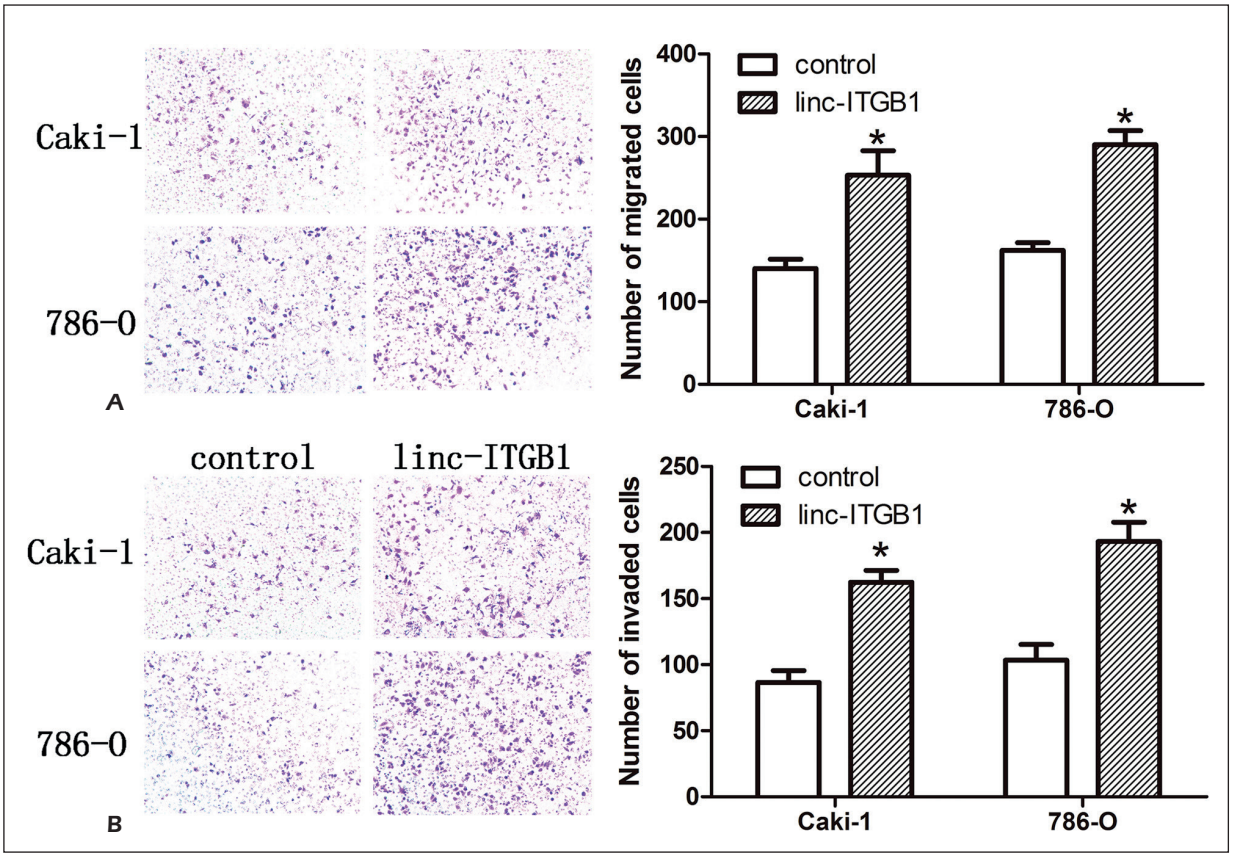

Figure 3. Overexpression of linc-ITGB1 promoted ccRCC cell migration and invasion. **A**, Transwell assay showed that the number of migrated cells was significantly increased after linc-ITGB1 overexpression in ccRCC cells (Magnification: 10×). **B**, Transwell assay showed that the number of invaded cells was significantly increased after overexpression of linc-ITGB1 in ccRCC cells (Magnification: 10×). The results represented the average of three independent experiments (mean ± standard error of the mean). *p<0.05, as compared with control cells. *p<0.05.

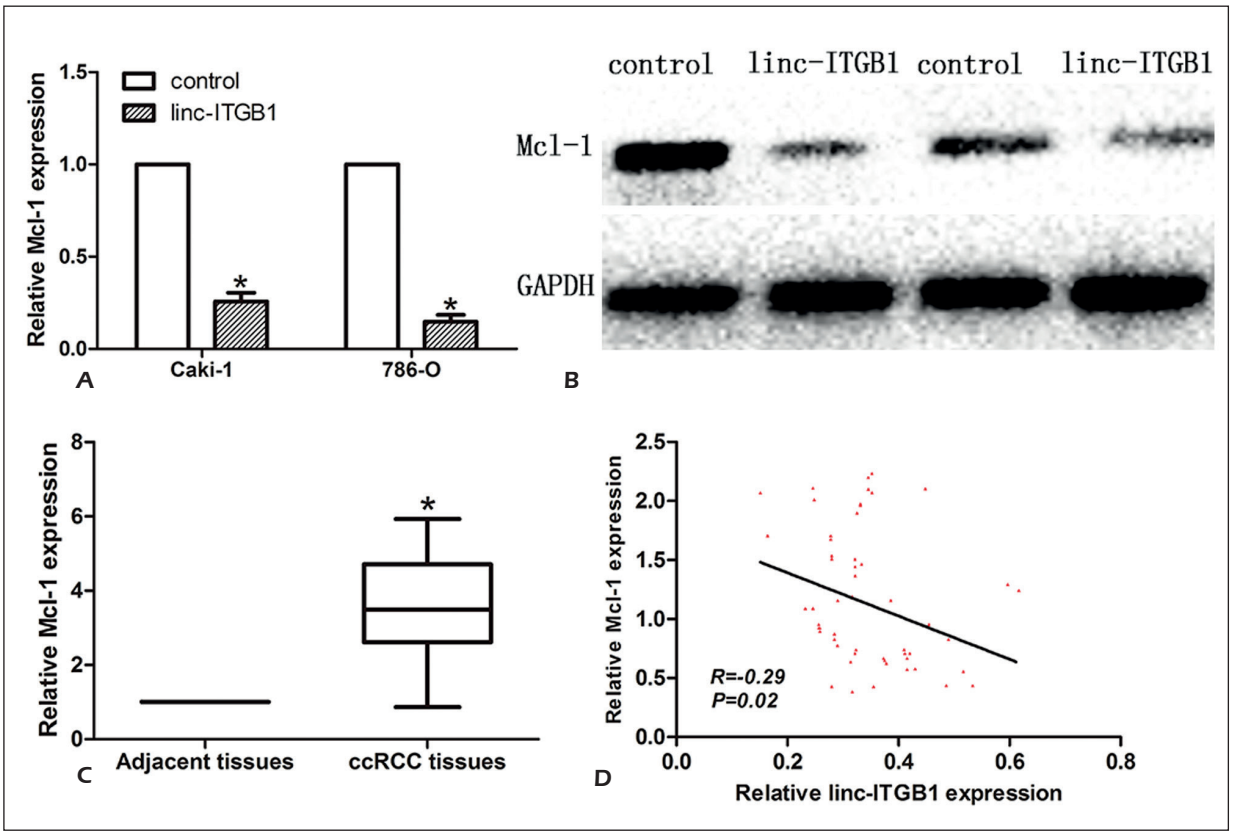

Figure 4. Interaction between linc-ITGB1 and Mcl-1. **A**, RT-qPCR results showed that Mcl-1 expression was significantly decreased in the linc-ITGB1 lentiviruses (linc-ITGB1) group compared with the empty vector (control) group. **B**, Western blot assay revealed that the protein expression of Mcl-1 was remarkably decreased in the linc-ITGB1 group compared with the control group. **C**, Mcl-1 was significantly downregulated in ccRCC tissues compared with adjacent tissues. D, Linear correlation between the expression level of Mcl-1 and linc-ITGB1 in ccRCC tissues. The results represented the average of three independent experiments. Data were presented as mean ± standard error of mean. *p<0.05.

progression of gastric cancer by suppressing the PI3K/AKT pathway[8]. LncRNA FAL1, serving as a potential oncogene for colon cancer, promotes the proliferation and inhibits the apoptosis of colon cancer cells[9]. LncRNA NBAT1 and lncRNA HOXC-AS3 play important roles in the development of gastric cancer[10,11]. Moreover, lncRNA ZNFX1 is significantly increased in hepatocellular carcinoma and gastric cancer, eventually leading to their development and progression[12].

Recently, evidence has revealed that multiple lncRNAs participate in the development of ccRCC. For example, long noncoding RNA DHRS4-AS1 functions as a tumor inhibitor in ccRCC[13]. The upregulation of lncRNA MALAT1 promotes the progression of ccRCC, which can be used as a potential prognostic biomarker and therapeutic target[14]. LncRNA SPRY4-IT1 is overexpressed in ccRCC. Moreover, it indicates poor prognosis of patients with ccRCC[15]. On the contrary, low expression of lncRNA NBAT-1 is associated with poor prognosis of ccRCC patients[16].

LncRNA linc-ITGB1, as a novel long noncoding RNA, has attracted more attention for its vital role in cancer progression. For instance, studies have proved that linc-ITGB1 promotes epithelial to mesenchymal transition and metastasis of hepatocellular carcinoma. This indicates that linc-ITGB1 may be a potential therapeutic target[5,6]. Migration and invasion of gallbladder cancer cells have been found to be remarkably inhibited after linc-ITGB1 knockdown[7]. In this study, we found that linc-ITGB1 was upregulated both in ccRCC samples and cells. Besides, a close relationship was observed between patients' prognosis and expression level of linc-ITGB1. Furthermore, the overexpression of linc-ITGB1 significantly promoted the migration and invasion of ccRCC cells. The above results indicated that linc-ITGB1 promoted tumorigenesis of ccRCC and might act as an oncogene.

Mcl-1 (myeloid cell leukemia 1) is an important member of the Bcl-2 family, functioning as a critical anti-apoptotic protein. For example, the inhibition of Mcl-1 facilitates the apoptosis of osteosarcoma cells induced by Pevonedistat[17]. Mcl-1 is an important contributor to bromine-domains and extra-terminal inhibitors resistance in hepatocellular carcinoma[18]. Mcl-1 promotes chemotherapy resistance in breast cancer by cooperating with MYC *via* proliferating cancer stem cells[19]. Recently, researchers have found that the overexpression of Mcl-1 promotes the development of lung cancer by suppressing cell apoptosis[20]. In the present study, we first discovered the interaction between Mcl-1 and linc-ITGB1. The results showed that the expression level of Mcl-1 was significantly downregulated after linc-ITGB1 overexpression. Furthermore, Mcl-1 expression in ccRCC tissues was positively related to linc-ITGB1 expression. All the above results suggested that linc-ITGB1 might promote tumorigenesis of ccRCC by regulating Mcl-1.

Conclusions

Our results identified that linc-ITGB1 was remarkably upregulated in ccRCC, which was negatively related to disease-free survival time of ccRCC patients. Besides, linc-ITGB1 could facilitate the migration and invasion of ccRCC cells by downregulating Mcl-1. These findings suggested that linc-ITGB1 might contribute to the treatment of ccRCC as a candidate target.

Conflict of Interests

The authors declare that they have no conflict of interest.

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