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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 MATERIALS: Linc-ITG[illegible] pression in both ccRCC cells and tissue sa[illegible]s was detected by Real Time-quantitative [illegible] merase Chain Reaction (RT-qPCR). Moreo[illegible] the association between linc-IT[illegible] expressi[illegible] level and patients' disease-[illegible]ival rat[illegible] was explored. Then, woun[illegible]ealing [illegible]d tran-swell assays were condu[illegible]. Furth[illegible]ore, the underlying mechanism [illegible]explo[illegible] RT-qPCR and Weste[illegible]lot[illegible]

RESULTS: Linc-[illegible]B1 ex[illegible]on level in ccRCC samples [illegible]markedly [illegible] than that of the adjacen[illegible] The expres[illegible] of linc-ITGB1 was c[illegible]y [illegible]d to the d[illegible]ease-free survival ti[illegible] of ccRCC[illegible]nts. Moreover, the migration[illegible]d invasion [illegible]RCC cells were remark[illegible] enhanced after [illegible]TGB1 upregulation *in vitro*. In[illegible]dition, the mRNA and protein ex[illegible]ion of[illegible]-1 were significantly downregulate[illegible]r[illegible]-ITGB1 [illegible]erexpression. Furthermore, [illegible]pressio[illegible]vel of Mcl-1 was negatively co[illegible]d w[illegible]he linc-ITGB1 expression in[illegible]CC ti[illegible]

C[illegible]CLUSIO[illegible] Our findings suggested that lin[illegible]B1 could enhance ccRCC cell migration [illegible]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

[illegible]enal cell ca[illegible]oma (RCC) is the most com-m[illegible]idney carc[illegible]ma, ranking the sixth leading cau[illegible]cane[illegible]lated death in America. Males are mo[illegible] to be diagnosed with RCC, with a [illegible]atio of 1.6:1.0 between males and females. Most [illegible] are diagnosed at the age between 40 to 70 [illegible]a[illegible][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 w... thesized and cloned into a pLenti-EF1a-... F2A-Puro vector (Biosettia Inc., San Diego, ... USA). Linc-ITGB1 lentiviruses (linc-ITGB1) ... empty vector (control) were p... in 293... cells. Subsequently, they w... ted into ccRCC cells. 48 h later, ... Time-... ntitative Polymerase Chain Reacti... T-qPC... to detect the linc-ITG... ex... ever... transfected cells.

RNA Extract... and RT-qPC...

Total ... was extra... according to the instruct... of TRIzol reagen... vitrogen, Carlsbad, ... USA)... tracted total RNA was reverse tra... ed i... omplementary deoxyribose nucleic ... NA) t... gh the reverse Tran...iption ... (TaKa... Biotechnology Co., Ltd., D... Chi... hermal cycle was as follows: 30... at 95... sec for 40 cycles at 95°C, 35... 60°C. Primer sequences used in this ... follows: Linc-ITGB1, F: 5'-AAC-AGGCCCCTCCTTACTC-3', R: 5'-GATGT-CCGAAGGCTAGGA-3'; Mcl-1, F: 5'-GTG-... GAACATCCTCGACTG-3', R: 5'-CGTG-TATTGCGTGGAGTCG-3'; U6: F: 5'-GCTTC-GGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Western Blot Analysis

Total protein was extracted from c... dioimmunoprecipitation assay (RIP... eyotime, Shanghai, China). The concentrati... f extracted protein was detected by the BCA ... in assay kit (TaKaRa, Dalian, China). Subseq... the protein samples were separ... by sodium ... sulphate-polyacrylamide ... electro... oresis (... PAGE) and transferre... to polyvinylidene difluoride (PVDF) me... br... (M... pore, B... rica, MA, USA). The ... mbran... re then ... ubated with the corresponding pri... and secondary antibodies. ... nti-GAPDH ... rabbit anti-Mcl-1 w... pro... by Cell Signaling Technology (CST; Danvers, M... SA), as well as goat anti-ra... ondary antib... mage J software was a... d for the assessment of protein expression.

...und Healing Assay

...er seeded ... 6-well plates, the cells were cult... in DM... l medium overnight. Then, the cells w... tched with a 200 μL plastic tip, followed by culture in serum-free DMEM. Each ... was repeated in triplicate independently. ... and 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-

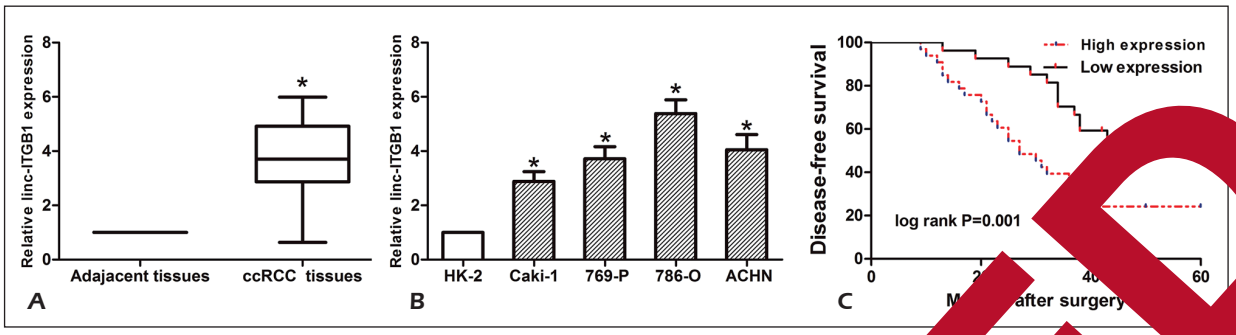


Figure 1. Expression of linc-ITGB1 in ccRCC tissues and cell lines. **A**, Linc-ITGB1 expres[illegible] ccRCC [illegible]ues was signi-cantly increased when compared with adjacent tissues. **B**, The expression levels of linc-IT[illegible]latix[illegible]p-actin [illegible]human ccRCC cell lines and 16HBE (normal human bronchial epithelial cell) were determined [illegible]RT-q[illegible]higher l[illegible]f linc-ITGB1 was associated with worse disease-free survival of ccRCC patients. Data we[illegible]sented a[illegible]± sta[illegible]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-[illegible] method were performed for analysis. p<0.[illegible] considered statistically significant.

Results

Linc-ITGB1 Expression[illegible]el in ccRCC Tissues and [illegible]

The linc-ITGB1 [illegible]xpr[illegible]60 [illegible]e samples and 4 cc[illegible] cell l[illegible]as detected *via* RT-qPCR. A[illegible]sult, linc-I[illegible] was significantly upre[illegible]t[illegible] ccRCC tis[illegible] samples (Figure 1A). [illegible]anwhi[illegible]c-ITGB1 expression level in c[illegible]C cells was [illegible]kably higher than that of [illegible]2 cells (human k[illegible]y epithelial cell) (Figu[illegible]B).

High[illegible]ession[illegible]Linc-ITGB1 [illegible]s Co[illegible]ted [illegible]n Worse [illegible]se-Fr[illegible]val of ccRCC Patients

[illegible] survival[illegible]e of ccRCC patients after surge[illegible]nalyzed by the Kaplan-Meier method. [illegible]ord[illegible]the median expression, 60 ccRCC [illegible]tients were divided into two groups: the high-[illegible]ITGB1 group and the low-linc-ITGB1 group. T[illegible]aplan-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 o[illegible]c-ITGB1 Pro[illegible]Migration[illegible]Invasion o[illegible]RCC Cells

[illegible]n this study, Caki-1 and 786-O ccRCC cell [illegible] were chose[illegible]r linc-ITGB1 overexpression. T[illegible]the linc-IT[illegible]1 expression in the transfected c[illegible]as c[illegible]med by qRT-PCR (Figure 2A). Moreov[illegible]nd healing assay results indicated that the overexpression of linc-ITGB1 signifi-[illegible]nhanced the migration ability of ccRCC [illegible]gure 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

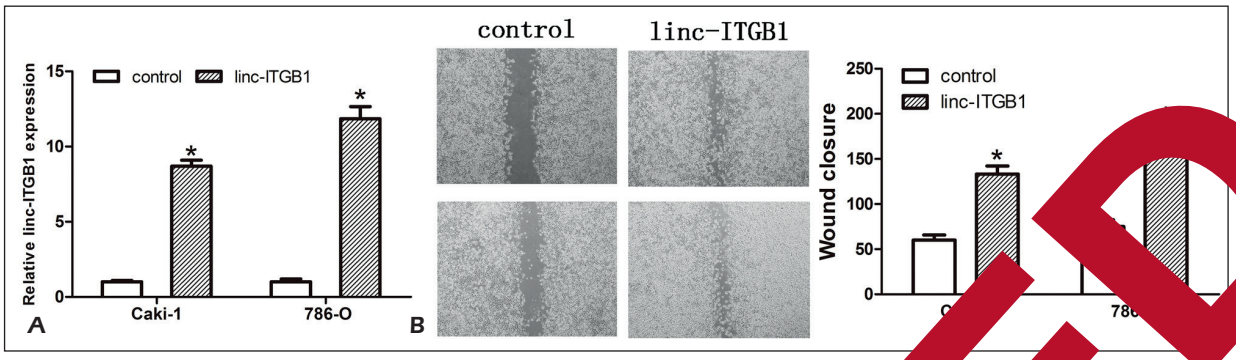
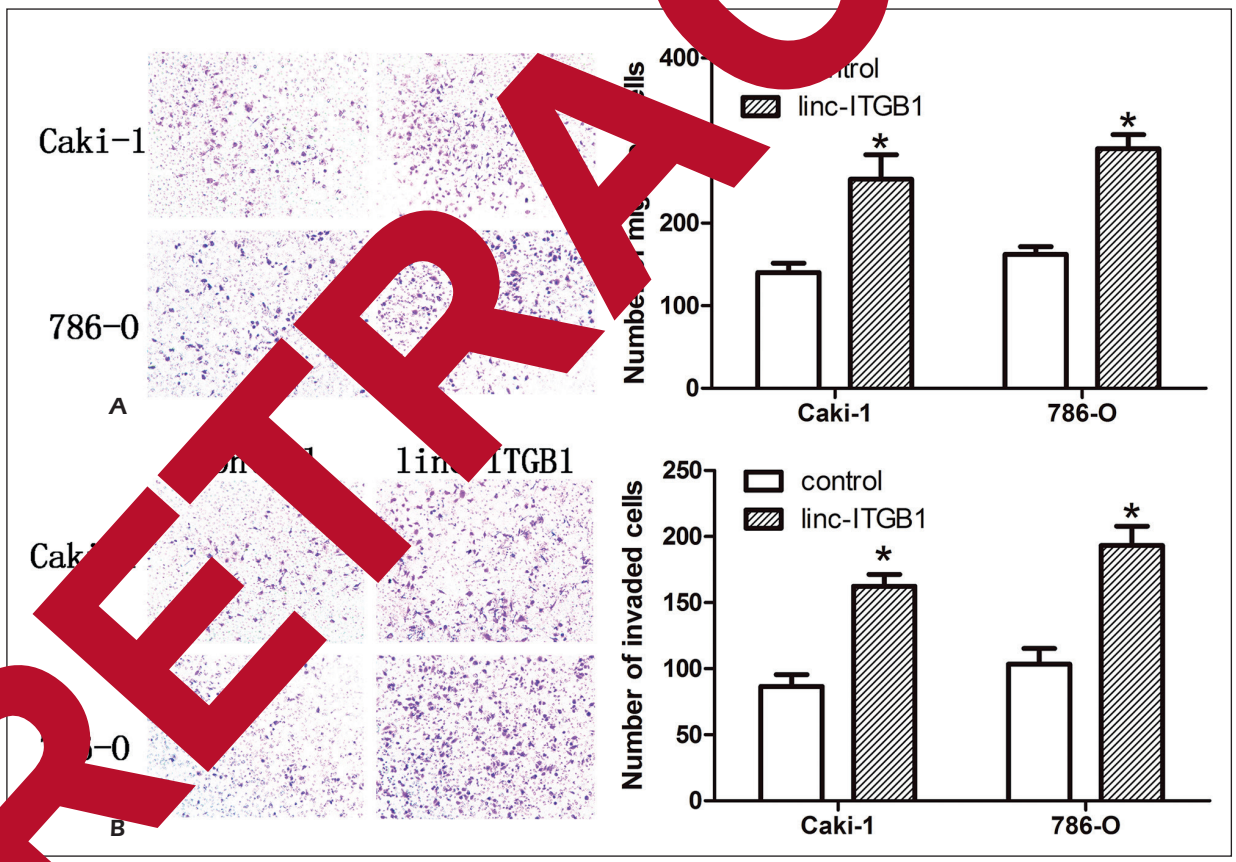


Figure 2. Overexpression of linc-ITGB1 promoted ccRCC cell migration. **A**, Linc-ITGB1 ... RCC ce... trans-fected with linc-ITGB1 lentiviruses (linc-ITGB1) and empty vector (control) was dete... by ... β-actin ... sed as an internal control. **B**, Wound healing assay showed that the overexpression of linc-... signifi... increa... migration of ccRCC cells (Magnification: 10×). The results represented the average of three i... endent experi... ± 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... of biologi... rocesses, including p... eration, migration an... metastasis of cancer. ... example, lncRNA HORAIRM1 inhibits the



...3. Overexpression of linc-ITGB1 promoted ccRCC cell migration and invasion. **A**, Transwell assay showed that the nu... 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.

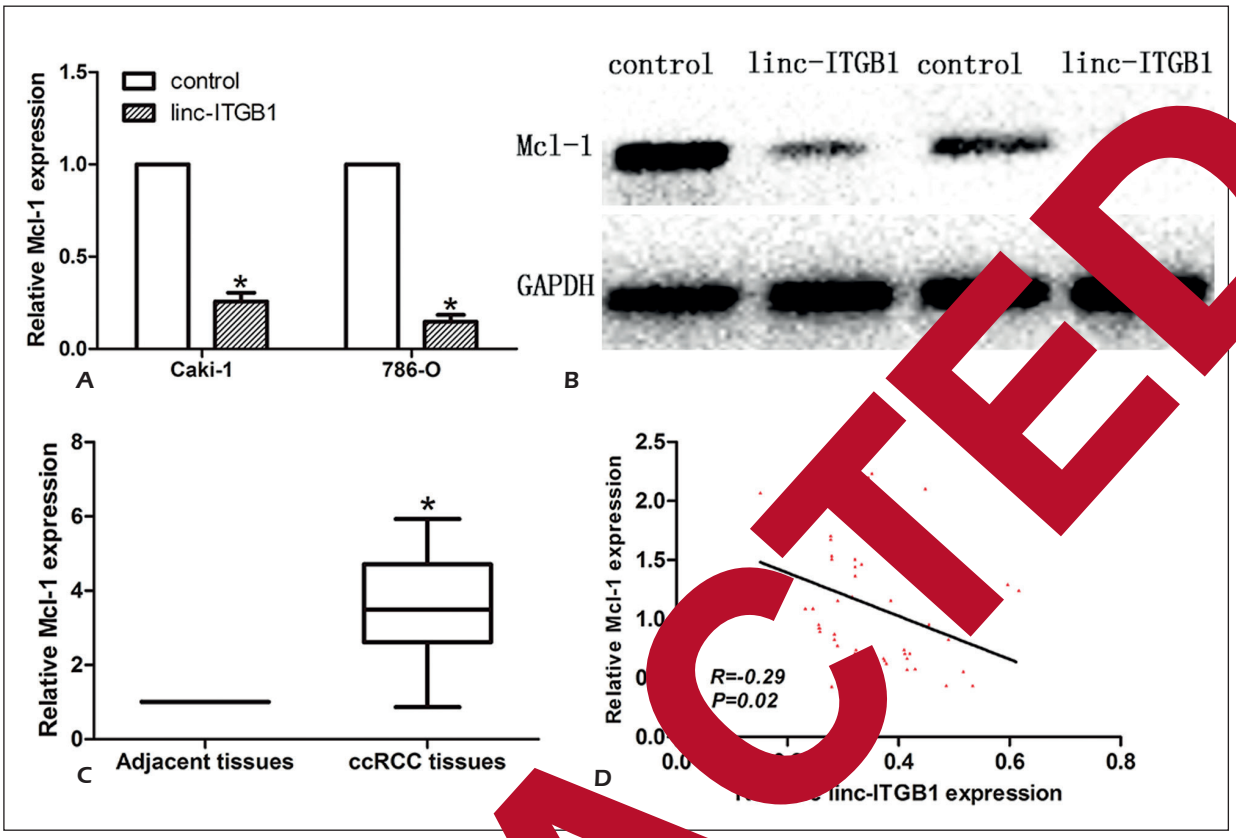


Figure 4. Interaction between linc-ITGB1 and Mcl[illegible] RT-[illegible] showed that Mcl-1 expression was significantly decreased in the linc-ITGB1 lentiviruses (linc-ITGB1[illegible] [illegible]pared with the empty vector (control) group. **B**, Western blot assay revealed that the protein expression of Mcl-[illegible] [illegible]markably decreased in the linc-ITGB1 group compared with the control group. **C**, Mcl-1 was signif[illegible]wnregula[illegible] ccRCC tissues compared with adjacent tissues. D, Linear correlation between the expression lev[illegible] linc-ITG[illegible] ccRCC tissues. The results represented the average of three independent experiments. Data w[illegible]resente[illegible]mean ± st[illegible]d error of mean. *p<0.05.

progression of gast[illegible]ancer t[illegible]pressing the PI3K/AKT pathw[illegible]ncRNA F[illegible]serving as a potential on[illegible]colon canc[illegible]promotes the proliferati[illegible]and in[illegible]the apoptosis of colon cance[illegible]ls[9]. LncRN[illegible]AT1 and lncRNA HOXC[illegible] play important[illegible]s in the develop[illegible]f gast[illegible]ancer[10,11]. Moreover, lncRNA ZN[illegible]is si[illegible]icantly increased in hepatocellular[illegible]a and[illegible]ric cancer, eventually [illegible]ding t[illegible]r dev[illegible]ment and progression[12].

[illegible]ently,[illegible]e has revealed that multipl[illegible]ncRNAs[illegible]rticipate in the development of[illegible]C. For example, long noncoding RNA [illegible]functions as a tumor inhibitor in [illegible]RCC[13]. The upregulation of lncRNA MALAT1 [illegible]notes the progression of ccRCC, which can b[illegible]d 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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