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Knockdown of long noncoding RNA linc-ITGB1 suppresses migration, invasion of hepatocellular carcinoma via regulating ZEB1

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Abstract. – OBJECTIVE: This research focuses on the influence of linc-ITGB1 on the metastasis of hepatocellular carcinoma and further explores its underlying mechanism.

PATIENTS AND METHODS: A total of 70 hepatocellular carcinoma patients were chosen for our study. RT-qPCR was used for detecting the expression level of linc-ITGB1 in their cancer tissues. Moreover, the expression level of linc-ITGB1 was also detected in hepatocellular carcinoma cell lines. Furthermore, whether linc-ITGB1 could affect the migrated and invaded ability of hepatocellular carcinoma cells was determined by wound healing assay and transwell assay. We further explored the potential mechanism by RT-qPCR and Western blot assay.

RESULTS: Linc-ITGB1 expression level in hepatocellular carcinoma tissues was remarkably higher than that in adjacent tissues. Moreover, migrated and invaded ability of hepatocellular carcinoma cells was inhibited through knockdown of linc-ITGB1. Further study revealed that linc-ITGB1 inhibited the expression of ZEB1 and then suppressed epithelial to mesenchymal transition (EMT), which is important during the metastasis of hepatocellular carcinoma. Moreover, the inhibition of cell invasion by silenced linc-ITGB1 could be rescued through overexpression of ZEB1 in hepatocellular carcinoma.

CONCLUSION: The results indicate that linc-ITGB1, a novel oncogene in tumorigenesis, could promote the metastasis and EMT via ZEB1, which may provide a possible therapeutic target in hepatocellular carcinoma.

Key words: linc-ITGB1, Hepatocellular carcinoma, ZEB1, EMT.

Introduction

Liver carcinoma, as a common cancer worldwide, has a high mortality rate, which ranks the second among malignant cancers. Most primary liver cancers are hepatocellular carcinoma worldwide[1]. The incidence of liver cancer in China accounts for more than half of the whole world. Although numerous treatments are available for hepatocellular carcinoma patients, high rate of recurrence and metastasis remains an intractable problem[2]. Therefore, further exploring the mechanisms involved in the metastasis of hepatocellular carcinoma is urgently required.

Recently researchers have confirmed the fact that dysregulated non-coding RNAs identified in many cancers are probably associated with many known oncogenes. As a subtype of noncoding RNA, long non-coding RNAs (lncRNAs) have a great function in tumor initiation, proliferation and metastasis in various cancers[3-5]. Moreover, the function of lncRNA in liver cancer becomes more important and has been frequently studied. For example, the expression of lncRNA HOTTIP/HOXA13 has a significant correlation with progression of hepatocellular carcinoma, which can be used for predicting disease outcome[6]. Moreover, in those hepatocellular carcinoma patients who receive liver transplantation, the cancer recurrence can be predicted according to lncRNA MALAT-1 expression[7]. LncRNA SNHG1 acts as an oncogene in hepatocellular carcinoma and promotes tumorigenesis[8]. During the metastasis of hepatocellular carcinoma, upregulated lncRNA ZFAS1 promotes migration of tumor cells[9]. Therefore, it is meaningful to find novel markers for diagnosis and treatment of hepatocellular carcinoma.

Epithelial to mesenchymal transition (EMT) has been identified as a key step of invasion and metastasis in epithelial cancers. More evidence suggests that lncRNAs could affect the migrated and invaded ability of cancer cells through regulating EMT. For example, in hepatocellular car-

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cinoma, EMT is induced by lncTCF7 after IL-6/STAT3 is transactivated[10]. LncRNA HOTAIR can down-regulate RNA binding motif protein 38 and then induce EMT in liver cancer cells[11]. LncRNA AOC4P functions as a tumor suppressor during liver cancer metastasis and downregulates Vimentin, which is an important marker of EMT[12]. A recent study[13] revealed that gallbladder cancer cell migration and invasion is remarkably inhibited after linc-ITGB1 is knocked down. However, up to now, a few literature has uncovered the role of linc-ITGB1 during hepatocellular carcinoma metastasis as well as its molecular mechanism.

Our present work revealed that linc-ITGB1 was higher in hepatocellular carcinoma tissue samples and cells. Silenced linc-ITGB1 inhibited the migrated and invaded ability of hepatocellular carcinoma *in vitro*. In addition, we further explored how linc-ITGB1 functions on EMT process as well as underlying mechanism in hepatocellular carcinoma cells.

Patients and Methods

Patients and Specimens

Cancer tissue specimens and adjacent t[illegible]s were acquired from 70 hepatocellular carcin[illegible] patients who received surgery at Yantaishan H[illegible] spital. No patients received ra[illegible]y or ch[illegible] motherapy before operation[illegible] hepatocellular carcinoma tissues were st[illegible] at -80°[illegible] n experienced pathologist asse[illegible] clin[illegible] written informed co[illegible]t wa[illegible]able for this study. The invest[illegible]on was c[illegible]med to the requirements of [illegible]ics Comm[illegible]f Yantaishan Hospital.

Cell C[illegible]e and Cultur[illegible]ndition

Th[illegible]stitute [illegible]Biochemistry and Cell Biology, Ch[illegible] Aca[illegible]y of Science (Shanghai, China) provi[illegible]n Hep[illegible]Bel-7402, 293T and L02 [illegible]mal [illegible]epithe[illegible]cell). Culture medium was c[illegible]t of [illegible] penicillin (Invitrogen Life Te[illegible]ologies, [illegible]sbad, CA, USA), 10% fetal bo[illegible]m (FBS) and Dulbecco's modified Ea[illegible]'s [illegible] (DMEM; Thermo Fisher Scientific, [illegible]c., Waltham, MA, USA). Besides, humidified [illegible]bator was maintained at 37°C with 5% CO_2. L[illegible]viral small hairpin RNA (shRNA) targeting linc-ITGB1 was synthesized and then cloned into the pLenti-EF1a-EGFP-F2A-Puro vector (Biosettia Inc., San Diego, CA, USA). Then, 293T cells were chosen for packaging the viruses, the linc-ITGB1 lenti-viruses (sh-linc-ITGB1) and the empty vector (sh-ctrl). For overexpression of ZEB1, a lentivirus targeting ZEB1 was synthesized and cloned into the pLenti-EF1a-EGFP-F2A-Puro vector (Biosettia Inc., San Diego, CA, USA). Next, 293T cells were chosen for pack[illegible] viruses, the ZEB1 lenti-viruses (pLV[illegible]B1) and the empty vector (pLV-ctrl). Foll[illegible]g are the sequences used for lentiviral vector [illegible]uction: linc-ITGB1, 5'-GCAGC[illegible]TTTC[illegible]AA-TATTGCTCGAGCAAT[illegible]CTGGA[illegible]A-GCTGC-3'; control, 5'-[illegible]GGAG[illegible]GTTTC-AGAATATCTCGAG[illegible]TTC[illegible]CAAACC-CTCCGCTTTTTT[illegible].

RNA Extrac[illegible]and RT-q[illegible]

As repo[illegible]o[illegible] manufactu[illegible] instructions, TRIzol reag[illegible]t (Invi[illegible] Carlsbad, CA, USA) was utilize[illegible]xtracting t[illegible]al RNA in these cells. cD[illegible] we[illegible]e synthesized [illegible]everse Transcription K[illegible]TaKaRa Biotechnology Co., Ltd., Dalian, Liaoning, China). We [illegible]formed RT-qPCR on ABI 7500 sy[illegible] (Applied [illegible]ystems, Foster City, CA, USA). Pri[illegible]for RT-[illegible]R are the following: linc-ITGB1, forwar[illegible]CTCAGCCTCCAGCGTTG-3' and reverse 5'-TGCTCTTGCTCACTCACACTCC-3'; [illegible] forward 5'-AAGTGGCGGTAGATGGTA-[illegible] and reverse 5'-AAGGAAGACTGATG-GCTGAAAT-3'; E-cadherin, forward 5'-TCTG-GAAGGAATGGAGGAGTC-3' and reverse 5'-AATTGGGCAAATGTGTTCAGC-3'; Vimentin, forward 5'-ATTCCACTTTGCGTTCAAGG-3' and reverse 5'-CTTCAGAGAGAGGAAGCCGA-3'; GAPDH, forward 5'-GAAGGTGAAGGTCGGA-GTC-3' and reverse 5'-GAAGATGGTGATGG-GATTTC-3'. The thermal cycle was as follows: 30 s at 95°C, 5 s at 95°C for 40 cycles, 35 s at 60°C.

Wound Healing Assay

5×10^5 of these cells were transferred into six-well plates overnight with the medium containing DMEM and FBS. When grown to 80% confluence, the cell layer was taken out for assay. After we scratched the cell layer with a plastic tip, the culture medium was replaced with DMEM. Wound closure was viewed at different time points. Each assay was independently repeated in triplicate.

Transwell Assays

After digested by trypsin/EDTA solution (Thermo Fisher Scientific, Inc., Waltham, MA, USA), cells were washed using serum-containing DMEM medium. For the invasion assays, the upper chamber of an insert (8 μm pore size;

Millipore, Billerica, MA, USA) was coated with 50 μg Matrigel (BD Biosciences, Franklin Lakes, NJ, USA), before it was added with 200 μL serum-free DMEM medium containing 5×10^4 cells. The lower chamber was added with DMEM medium containing 10% FBS. 24 h later, upper surface of chambers was wiped with cotton buds. Then, the chambers were immersed in 100% precooling methanol (Sigma-Aldrich, St. Louis, MO, USA) for 10 min, stained with 0.05% crystal violet (Sigma-Aldrich, St. Louis, MO, USA) for 30 min and rinsed in phosphate-buffered saline (PBS). Pictures of these cells were obtained from light microscopic (DFC500; Leica, Wetzlar, Germany). The data for migration and invasion were counted from three fields per membrane. Each test was independently repeated thrice.

Western Blot Analysis

A protein assay, bicinchoninic acid method (BCA) (Beyotime, Shanghai, China) was utilized for quantifying the total protein expression. The target proteins were replaced to the polyvinylidene fluoride (PVDF) membrane, which was then blocked in 5% dry milk at 37°C for 1 h af it was fractionated by sodium dodecyl te-polyacrylamide gel electrophoresis (SD A-GE). Following, immunostaining with antibo (Cell Signaling Technology, CST, Danvers, M USA) overnight at 4°C, 1:1000 nti-E-c dherin, 1:1000 rabbit anti-Vi in, 0 rabbi anti-ZEB1, and 1:5000 ra anti-G H were used. PBS supplemented w 1% utilized 4 times to the anes, which were then cultivated within 1:1000 goat anti-rabbit secondary antibody. 1 h later, PBS was again used to wash the membranes three times for 15 min. The compare between relevant protein levels was conducted by ImageJ software.

Statistical Analysis

Data was analyzed accor ed with SPSS.18.0 (SPSS Inc., Chicago, IL, Quantitative data was presented as an ± raph PAD 4.0 (GraphPad Softw Inc., La Jo A USA) helped presenting e cons uences. method of $2^{-\Delta\Delta CT}$ was o me e the relati e expression of mR ndent ples *t*-test was chose the m 1. On < 0.05 could be cons l of statisti

Results

Higher Level of Linc-ITGB1

igher level inc-ITGB1 was monitored in h cellular c noma tissues and cell lines. Fir qPCR s performed in 70 pairs of hepatoce noma tissues and adjacent tissues for detecting linc-ITGB1 expression. As the result, linc-ITGB1 levels were monitored in hepa- carcinoma tissue samples (Figure 1A). We further found that the hepatocellular carcinoma cells had increased expression of linc-ITGB1 compared with L02 (Figure 1B). Moreover, we correlated linc-ITGB1 expression with clinicopathological features in those hepatocellular carcinoma patients, and found that high expression of

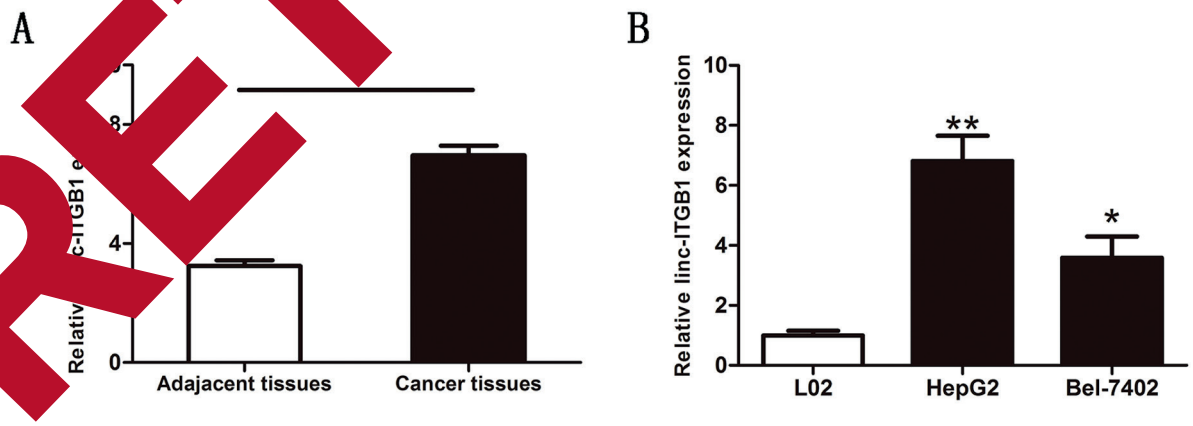

Figure 1. Expression levels of linc-ITGB1 were increased in hepatocellular carcinoma tissues and cell lines. ***(A)*** Linc-ITGB1 expression was significantly increased in the hepatocellular carcinoma tissues compared with adjacent tissues. ***(B)*** Expression levels of linc-ITGB1 were determined in the human hepatocellular carcinoma cell lines and normal liver epithelial cell (L02) by RT-qPCR. Data are presented as the mean ± standard error of the mean. *$p < 0.05$.

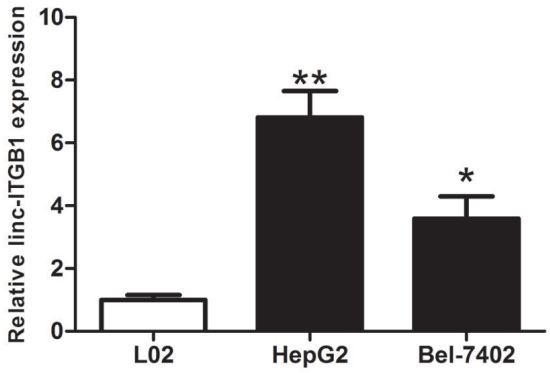

Figure 2. The expression of linc-ITGB1 in HepG2 cells transduced with control shRNA vector (sh-ctrl) or linc-ITGB1 shRNA (sh-linc-ITGB1) was detected by RT-qPCR. Data are presented as the mean ± standard error of the mean. *$p < 0.05$.

linc-ITGB1 was associated with aggressive BCLC stage and large tumor size (Table I).

Knockdown of linc-ITGB1 Suppressed Aggressiveness of Hepatocellular Carcinoma Cells

Since HepG2 has the highest ession level of linc-ITGB1, we chose Hep ells for knockdown of linc-ITGB1 ich w alled sh-linc-ITGB1. Besides, s l referred he cells transfected by a co sequ (Figu Knockdown of linc- l su sed cell mi- gration in sh-linc-I B1 mpared h sh-ctrl cells (Figur). In pa tran assay demonstrated umbers of ells were

Table I. Correlation between linc-ITGB1 expression and clinicopathol acteristics in hep ular carcinoma patients.

		Expr on of linc-ITG		
Characteristics	**Patients**	**Low expressi**	**High ression**	***p*-value**
Total	70	31	39	
Age (years)				0.728
> 60	30		16	
≤ 60	40	17	23	
Gender				0.606
Male	43		25	
Female	27		14	
HBsAg				0.767
Positive			20	
Negative	3	14	19	
Liver cirrhosis				0.681
With		19	22	
Without	29	12	17	
AFP (ng/mL)				0.402
> 20	39	19	20	
≤ 20	31	12	19	
Tumor siz				0.007
> 5		9	24	
≤ 5		22	15	
Tu umber				0.538
Sing	31	15	16	
Multip	39	16	23	
psula				0.350
ete	34	17	17	
N	36	14	22	
P tage				0.008
	42	24	18	
-C	28	7	21	
umor differentiation				0.810
/moderate	35	16	19	
	35	15	20	
Vascular invasion				0.978
Present	34	15	19	
Absent	36	16	20	

HBsAg: hepatitis B surface antigen; AFP: alpha-fetoprotein; BCLC: Barcelona Clinic Liver Cancer.

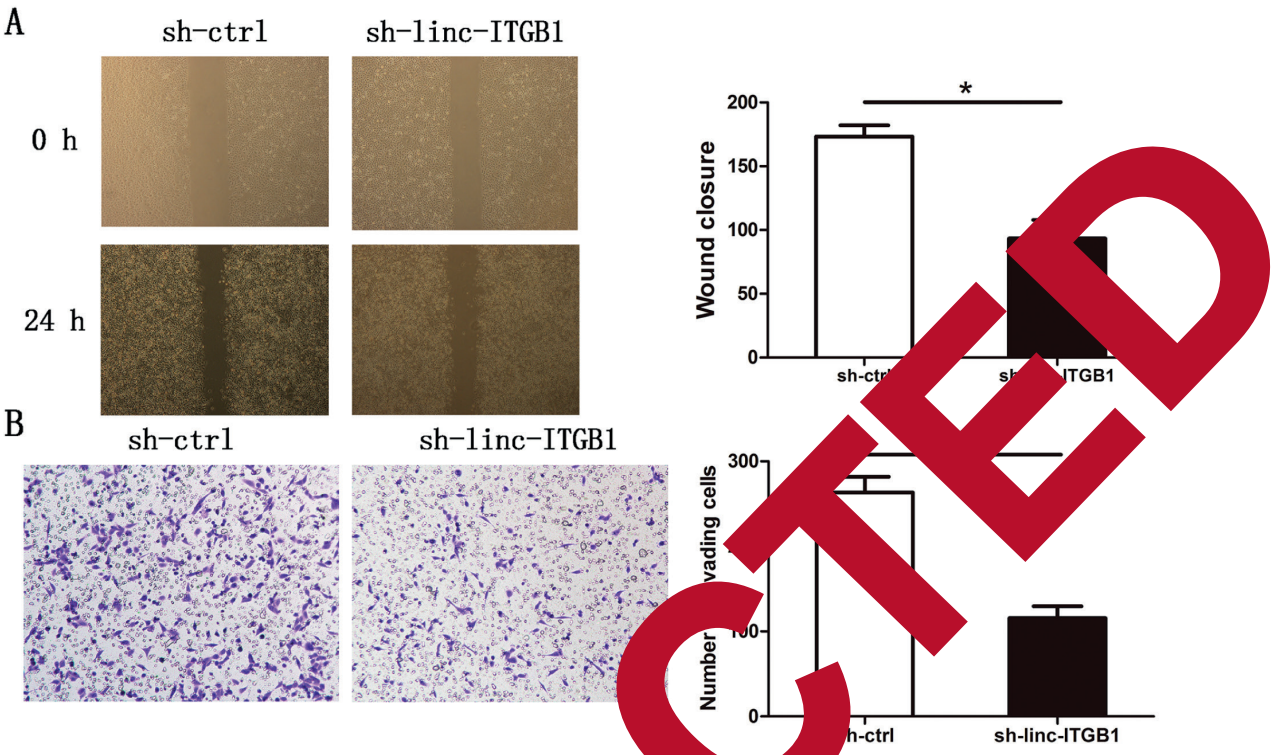

Figure 3. Knockdown of linc-ITGB1 inhibited migr[illegible]nd invasion o[illegible]s. ***(A)*** Wound healing assay showed that knockdown of linc-ITGB1 significantly suppressed [illegible] in sh-linc-ITGB1 cells compared with sh-ctrl cells. ***(B)*** Transwell assay demonstrated that the number of invade[illegible]s w[illegible]antly decreased in sh-linc-ITGB1 cells compared with sh-ctrl cells. The results represent the average of three [illegible]ende[illegible]s (mean ± standard error of the mean). *$p < 0.05$.

reduced in sh-linc-ITGB1 ce[illegible]mpa[illegible]with sh-ctrl cells (Figure 3B).

Knockdown of li[illegible]GB[illegible]ited ZEB1 in Hepatocellul[illegible]arcinom[illegible]lls

Recent studi[illegible]revealed t[illegible]etastasis of cancers are [illegible]ated [illegible]MT process, which can be regulat[illegible]y long non[illegible]g RNA. ZEB1, as a vital role in inducing EMT process, was also chosen for our research. Therefore, we investigated whether linc-ITGB1 could function on EMT-related protein and ZEB1. Results of RT-qPCR showed that Vimentin and ZEB1 were decreased and E-cadherin was increased at mRNA level after linc-ITGB1 was silenced in HepG2 (Figure 4A). Moreover, Western blot analysis showed

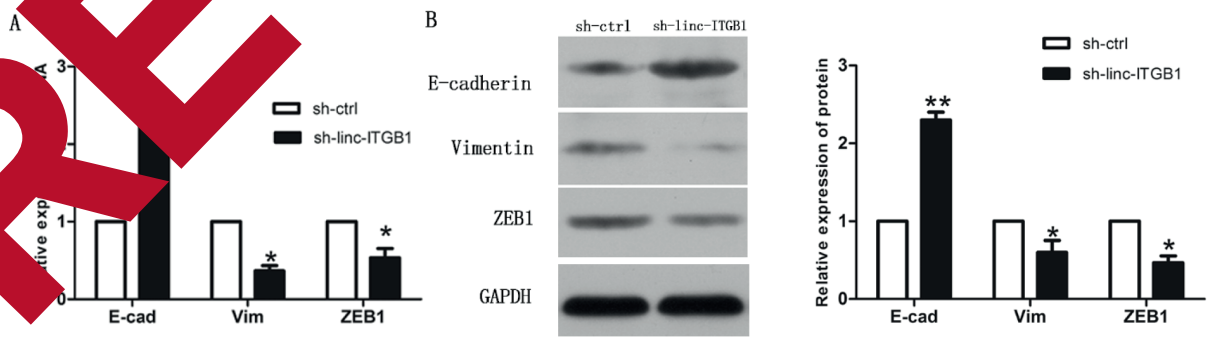

Figure 4. Knockdown of linc-ITGB1 suppressed EMT process via inhibiting ZEB1. ***(A)*** Knockdown of linc-ITGB1 influenced the mRNA of EMT-related protein and ZEB1 in HepG2 cells. ***(B)*** Knockdown of linc-ITGB1 influenced the protein level of EMT-related protein and ZEB1 in HepG2 cells. *$p < 0.05$.

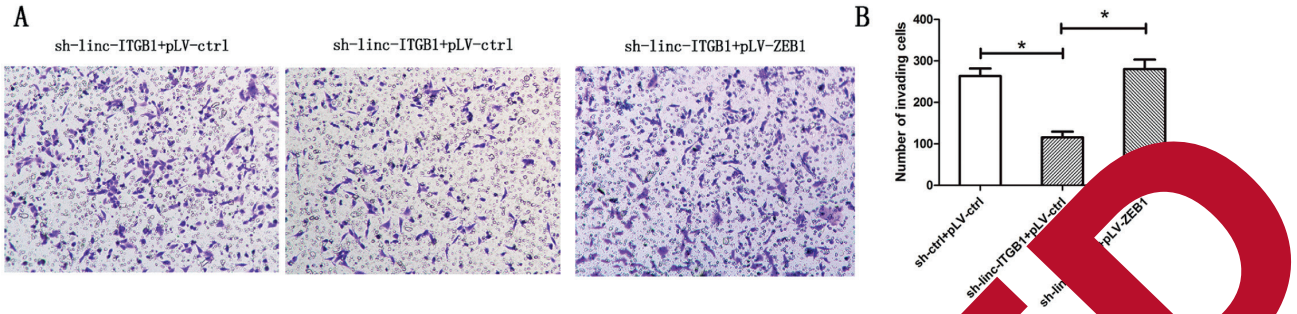

Figure 5. Rescue experiments showed that the inhibition of cell invasion by silenced linc-ITGB[illegible]d be rescue[illegible] overexpression of ZEB1 in hepatocellular carcinoma.

that Vimentin and ZEB1 were downregulated and E-cadherin was upregulated at protein level after linc-ITGB1 was silenced in HepG2 (Figure 4B). Moreover, rescue experiments revealed that the inhibition of cell invasion by silenced linc-ITGB1 could be rescued through overexpression of ZEB1 in hepatocellular carcinoma (Figure 5).

Discussion

Evidence has identified that lncRNAs [illegible] cipate in many important biological proc[illegible]s including cancer metastasis and tumorigen[illegible] which provide potential therapeutic targets [illegible] most cancers. A latest study[14] [illegible]at linc-ITGB1 enhanced tumor cell [illegible]ssiv[illegible]s in human breast cancer. In this [illegible]k, linc[illegible]GB1 was found higher expressed in [illegible] sa[illegible] ls of hepatocellular c[illegible]nom[illegible]s expression was associated wi[illegible]mor size [illegible]BCLC stage in patients. Fur[illegible] cell migra[illegible]and invasion were in[illegible]ed [illegible]patocellular carcinoma cells after [illegible]-ITGB1 w[illegible]nocked down. Data above s[illegible]est that linc-ITG[illegible]night serve as an onco[illegible] and p[illegible]mote the aggressiveness of hepa[illegible]ular c[illegible]noma.

EM[illegible] import[illegible]process in tumor metastasis, w[illegible]can h[illegible]gulated by lncRNA. The[illegible] we [illegible]xplored whether linc-ITGB1 co[illegible]regulate[illegible]T and then influence aggressiv[illegible] of hepatocellular carcinoma cells. Moreo[illegible], res[illegible] RT-qPCR and Western blotting assay demonstrated that the level of Vimentin was [illegible]eased and that of E-cadherin was increased o[illegible] linc-ITGB1 was silenced. Indeed, Vimentin is one of the mesenchymal phenotype cell biomarkers, while E-cadherin is one of the epithelial phenotype cell biomarkers. These data indicated that linc-ITGB1 made effect in hepatocellular carcinoma metas[illegible]via regulati[illegible]. However, it remaine[illegible]k[illegible] through [illegible] mechanism linc-ITGB1 influenc[illegible]e process of EMT.

Ac[illegible]ting evid[illegible]reveals that ZEB1 is fu[illegible]ental in tumorig[illegible]sis and metastatic b[illegible]viors. For instance, ZEB1 is upregulated in c[illegible]rectal cancer[illegible]d is remarkably related to poor p[illegible]osis[15]. Mor[illegible]er, a recent paper[16] shows that ZE[illegible]gulate[illegible]cadherin, a marker of EMT, in colore[illegible]. In addition, ZEB1 induces tumor metastasis and is associated with loss of cell [illegible]y in cancers[17]. A lately report discovers [illegible] acidic microenvironment-induced EMT is inhibited by miR-652 in pancreatic cancer, and ZEB1 plays a vital role in this process[18]. ZEB1 acts as an oncogene in many biological processes of many cancers, especially metastasis behavior. Lately, a new investigation[19] shows that in hepatocellular carcinoma, miR-139-5p suppresses EMT and metastasis of cancer cells through regulating ZEB1 and ZEB2. In our research, knockdown of linc-ITGB1 inhibited the expression of ZEB1, which was remarkably associated with EMT-related protein. Furthermore, rescue experiments revealed that the inhibition of cell invasion by silenced linc-ITGB1 could be rescued through overexpression of ZEB1 in hepatocellular carcinoma. Thus, we inferred that knockdown of linc-ITGB1 reversed EMT process via regulating ZEB1, which leads to changes in metastasis behavior of hepatocellular carcinoma cells.

Conclusions

We showed that linc-ITGB1 was higher expressed in hepatocellular carcinoma tissue samples and cells. Besides, silence of linc-ITGB1 could inhibit migration, invasion, and EMT process

of hepatocellular carcinoma through regulating ZEB1. These findings implied that linc-ITGB1 could act as a prospective therapeutic target for hepatocellular carcinoma.

Conflict of interest

The authors declare no conflicts of interest.

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