Long Non-coding RNA Linc-ITGB1 Knockdown Inhibits Cell Migration and Invasion in GBC-SD/M and GBC-SD Gallbladder Cancer Cell Lines

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Gallbladder cancer is a highly aggressive malignancy with a low 5-year survival rate. Despite advances in the molecular understanding of the initiation and progression in gallbladder cancer, treatment modalities such as surgery, radiotherapy, or chemotherapy in advanced cases did not yield promising outcomes. Therefore, it is of great importance to uncover new mechanism underlying gallbladder cancer growth and metastasis. In this study, we identified a differentially expressed long intergenic non-coding RNA, linc-ITGB1, in a pair of higher and lower metastatic gallbladder cancer cell sublines. Then, the potential role of linc-ITGB1 in gallbladder cancer cell proliferation, migration, and invasion was explored using a lentivirus-mediated RNA interference system. Functional analysis showed that knockdown of linc-ITGB1 significantly inhibited gallbladder cancer cell proliferation. Moreover, cell migration and invasion were reduced by over twofold in linc-ITGB1 knockdown cells probably due to upregulation of β-catenin and downregulation of vimentin, slug, and TCF8. In conclusion, linc-ITGB1 potentially promoted gallbladder cancer invasion and metastasis by accelerating the process of epithelial-to-mesenchymal transition, and the application of RNA interference targeting linc-ITGB1 might be a potential form of gallbladder cancer treatment in advanced cases.

Key words: epithelial-to-mesenchymal transition, gallbladder cancer, linc-ITGB1, metastasis, RNA interference

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As the most common malignancy in the biliary tract, gallbladder cancer (GBC) is an aggressive tumor with a 5-year survival rate of only 10% (1,2). The poor prognosis of GBC may result from the lack of severe symptoms, and thus, it is always diagnosed at an advanced stage (3). Resection is currently the only potentially curative treatment, but for unresectable GBC cases, the 5-year survival is minimal (3,4). It has previously been reported that radical surgery may help to improve overall survival rates for patients with GBC and the optimal surgical approach (e.g., simple cholecystectomy alone or additional locoregional lymph node dissection) may vary with the disease stage at the time of diagnosis (4–8). However, radiotherapy or chemotheraphy in advanced cases did not yield promising outcomes (9–11). Therefore, new effective therapeutic strategies are greatly needed, especially for advanced GBC cases.

Long non-coding RNAs (lncRNAs) are a class of newly identified non-coding RNA molecules that are longer than 200 base pairs in length, emerging as key regulators of gene expression and tumorigenesis in various cancer types, including colorectal cancers, hepatocellular carcinomas, lung cancers, blood neoplasms, and so on (12–21). LncRNAs can be classified into several subtypes including intergenic, sense, antisense, and bidirectional according to their genomic relationship with coding genes. Thus far, thousands of lncRNAs have been identified in decades by genomewide sequencing studies. Many of them have been proved to be functional and aberrantly expressed in various cancers, including ovarian cancer, breast cancer, and liver cancer (22,23). However, the implication of lncRNAs especially lincRNAs in GBCs has been rarely investigated.

Our previous report initially showed the involvement of a lncRNA in GBC, namely metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), which promoted the proliferation and metastasis of GBC cells by activating the ERK/MAPK pathway (24). To identify novel predictive biomarkers and effective therapeutic targets, in this study, we focused on a long intergenic non-coding RNA-ITGB1 (lincRNA-ITGB1) and investigated its expression and cellular functions in GBC cells. LincRNA-ITGB1 was differentially expressed in a pair of higher and lower metastatic GBC cell sublines (termed GBC-SD/M and GBC-SD, respectively (25)). The correlations of lincRNA-ITGB1 with GBC growth and metastasis were further examined via a lentivirus-mediated RNA interference (RNAi) system in GBC-SD/M cells.

Research Article
Materials and Methods

Cell culture
Human gallbladder cancer cell lines GBC-SD and GBC-SD/M were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), which represent a relative lower and higher metastatic subline, respectively (25). GBC-SD and GBC-SD/M cells were grown in DMEM (Gibco, Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS, Biowest, Kansas City, MO, USA), and 293 T cells were grown in DMEM (HyClone, Logan, UT, USA) supplemented with 10% FBS. All cells were cultured in a humidified incubator containing 5% CO2 at 37 °C.

Lentiviral vector construction and infection
The shRNA sequence targeting human linc-ITGB1 (TCONS_00018476) gene was obtained from LNCipedia (www.lncipedia.org/). The sequence was 5’-GCAGGCTTGGCAGAATATTGCTCGAGCAATATTCTGGAAACAGCTGC-3’ and control sequence was 5’-GCAGGAGGTGTTGAAAAAATATCTCGAGCAATATTCTGGAAACAGCTGC-3’. They were constructed into green fluorescent protein (GFP)-encoding pFH-L vectors at NheI/PacI restriction sites, respectively. The reconstructed vectors were then cotransfected with two packing vectors, pCMVΔR8.92 and pVSVG-I, into 293 T cells to generate lentiviruses. All three vectors were obtained from Hollybio Company (Shanghai, China). After 96 h of incubation, lentiviruses targeting linc-ITGB1 (named as shlinc-ITGB1) or control (named as shCon) were collected by purification and precipitation. For lentivirus infection, GBC-SD/M cells were cultured in 6-well plates with a concentration of 10^4 cells/well. Then, two lentiviruses (shlinc-ITGB1 and shCon) were applied to cultured cells at a multiplicity of infection (MOI) of 50. The plates were examined by fluorescence microscopy 96 h after incubation (×100 magnification). The infection efficiency was determined by counting the percentage of GFP-positive cells.

Quantitative real-time PCR
Total RNA was isolated from cultured cells using the Trizol reagent. For cDNA synthesis, 2 μg of total RNA was retrotranscribed for Mu-MLV (MBI Fermentas, Euromedex, Souffelweyersheim, France). Each PCR mixture (20 μL) contained 10 μL of 2X SYBR premix Ex Taq, 5 μL of the sample solution as the template, 0.8 μL of forward and reverse primers (2.5 μM), and ddH2O to make up the volume. The PCR was performed in the Bio-Rad connect real-time PCR platform using the following conditions: initial denaturation (95 °C, 1 min), 40 cycles of denaturation (95 °C, 5 s), and annealing extension (60 °C, 20 s), and 20 cycles of extension (72 °C, 20 s). The expression of selected genes was calculated using the 2^-ΔΔCt method. The primers used for qRT-PCR were as follows: linc-ITGB1: 5’-CTTCTCAGGCTCCAGGGTT (forward) and 5’-TGCT CTTGCTCACCTGAG-3’ (reverse), actin: 5’-GTTGAC ATCCCAAGGAC-3’ (forward) and 5’-AAAGGGTGTAAC GCAACTA-3’ (reverse), E-cadherin: 5’-TCTGGAGAAGATG GAGGATGC-3’ (forward) and 5’-AATGGCGGAATGTGTT CAGC-3’ (reverse), beta-catenin: 5’-TGGTAGGGTAAAT CAGTAAGGG-3’ (forward) and 5’-CTTGAAGCATTGAT CACACGAC-3’ (reverse), and vimentin: 5’-ATTCCACCT TGCCTTCAAG-3’ (forward) and 5’-CTTCAGAGAGAGGA AGCCGA-3’ (reverse).

MTT viability assay
After transduction with shlinc-ITGB1 or shCon, GBC-SD/M cells were seeded in 96-well plates at a density of 2 × 10^3 cells/well. The cell proliferative rate was calculated using MTT method on 1, 2, 3, 4, and 5 days after lentivirus infection. Briefly, 20 μL of MTT solution was added into each well to incubate for 4 h followed by replacing with 100 μL of DMSO. The absorbance value at optical density (OD) 595 nm of each well was recorded.

Wound healing assay
After transduction with shlinc-ITGB1 or shCon, GBC-SD and GBC-SD/M cells were seeded in 96-well plates and cultured until approximately 90% confluence, respectively. The plates were then scratched linearly with 200-μl pipette tips and allowed to recover in serum-free medium at 37 °C. At indicated time-points, cells were observed under light microscope and fluorescence microscope (×40 magnification). Migration areas were analyzed for uncovered wound areas by an image analysis program (Image-Pro Plus, Media Cybernetics, Silver Spring, MD, USA).

Transwell assay
Cell migration and invasion assays were conducted using the transwell chambers and matrigel invasion chambers (Corning, NY, USA), respectively. For transwell chambers, cells were cultured into the upper chamber in 200 μL of serum-free medium with a concentration of 3 × 10^5 cells/well. Then, 500 μL of serum-rich medium was added to the lower chamber, and the plate was incubated for 6 h at 37 °C. For matrigel invasion chambers, cells were cultured into the upper chamber in 200 μL of serum-free medium with a concentration of 6 × 10^5 cells/well. Then, 500 μL of serum-rich medium was added to the lower chamber, and the plate was incubated for 16 h at 37 °C. For both assays, at indicated time-points, the upper surface of the filter was swabbed to remove the cells that did not migrate. The lower compartment was immersed with methanol, and then, migrated cells were stained with crystal violet. Excess crystal violet stain was washed off using distilled water. Cells were counted in five random fields under a light microscope (×100 magnification), and an average of cell count in each group was calculated. Besides, migrated cells were dissociated and lysed and the amount of crystal violet was quantified using an OD


Role of Linc-ITGB1 in Gallbladder Cancer

ATCCGCAAAGAC-3’ (forward) and 5’-AAAGGGTGTAAC GCAACTA-3’ (reverse), E-cadherin: 5’-TCTGGAGAAGATG GAGGATGC-3’ (forward) and 5’-AATGGCGGAATGTGTT CAGC-3’ (reverse), beta-catenin: 5’-TGGTAGGGTAAAT CAGTAAGGG-3’ (forward) and 5’-CTTGAAGCATTGAT CACACGAC-3’ (reverse), and vimentin: 5’-ATTCCACCT TGCCTTCAAG-3’ (forward) and 5’-CTTCAGAGAGAGGA AGCCGA-3’ (reverse).
570 nm reading on a spectrophotometer after dissolving with DMSO.

**Western blot analysis**

To detect the function of linc-ITGB1 in epithelial-to-mesenchymal transition (EMT), Western blot analysis was performed in GBC-SD/M cells after linc-ITGB1 knockdown using an EMT antibody sample kit (Cell Signaling Technology, Danvers, MA, USA). Whole cell extracts were prepared with ice-cold cell lysis buffer (2% mercaptoethanol, 20% glycerol, and 4% SDS, in 100 mM Tris–HCl buffer, pH 6.8), and the protein concentration was determined by the enhanced BCA protein assay kit (Beyotime, Shanghai, China). Then, protein samples were detected according to the protocol of CST. Following antibody was used: vimentin antibody (1:1000 dilution; BD company, Franklin Lakes, NJ, USA), TCF8/ZEB1 rabbit mAb (1:800 dilution; Cell Signaling Technology); slug rabbit mAb (1:1000 dilution; Cell Signaling Technology), and β-catenin rabbit mAb (1:800 dilution; Cell Signaling Technology).

**Statistical analysis**

All data were shown as mean ± standard deviation (SD) of at least three independent experiments. For statistical analysis, the Student’s t-test was applied. A p-value of <0.05 was considered statistically significant.

**Results**

**Knockdown of linc-ITGB1 by shRNA lentiviral system in gallbladder cancer cells**

We firstly detected the expression of linc-ITGB1 in two human GBC cell cultures, GBC-SD and GBC-SD/M, which represent a relative lower and higher metastatic subline, respectively. Liu et al. (25) established the subpopulation with high metastatic potential from an experimental liver metastatic model of GBC. Herein, as shown in Figure 1A, linc-ITGB1 expression was remarkably higher in GBC-SD/M cells than in GBC-SD cells, suggesting that linc-ITGB1 might be involved in GBC metastasis. To evaluate the potential role of linc-ITGB1 in human GBC progression, we then applied a lentivirus-mediated RNAi system to knock down linc-ITGB1 expression in GBC-SD/M cells. As revealed in Figure 1B, more than 80% of cells expressed GFP in GBC-SD/M cells infected with shlinc-ITGB1 or shCon, indicating a successful infection efficiency. Similarly, GBC-SD cells were also successfully transduced with shlinc-ITGB1 or shCon (Figure S1A). After 96 h of incubation, the relative expression level of linc-ITGB1 was significantly decreased in the shlinc-ITGB1 group with a knockdown efficiency of 97.1% as compared to control, as determined by qRT-PCR analysis (Figure 1C). This indicated that linc-ITGB1 expression could be specifically downregulated in GBC cells by RNAi.

**Linc-ITGB1 knockdown suppressed gallbladder cancer cell proliferation**

To investigate the effect of linc-ITGB1 on GBC cell proliferation, MTT assay was performed in GBC-SD/M cells 5 days after lentivirus infection. As revealed in Figure 2, the proliferative rate of the shlinc-ITGB1 group was markedly reduced by 60% on day 5, as compared to control. This indicated that knockdown of linc-ITGB1 could significantly suppressed the proliferation of GBC cells.

**Linc-ITGB1 knockdown inhibited gallbladder cancer cell migration**

To examine the contribution of linc-ITGB1 to GBC cell migration, we knocked down the expression of linc-ITGB1 and conducted two in vitro assays. The classical ‘wound healing’ scratch assay is a convenient model for evaluating cell motility and migration. Representative images of uncovered wound areas are shown in Figure 3A. As revealed in Figure 3B, at 24 and 36 h post-scratching, the area of...
occupancy was significantly smaller in the shlinc-ITGB1 group (24 h: 25.294 ± 5.631%, 36 h: 32.796 ± 7.312%) than in the control (24 h: 60.349 ± 2.839%, 36 h: 70.459 ± 4.192%), indicating that linc-ITGB1 depletion had a strong inhibitory effect on the capacity of cells to repair a ‘wounded’ monolayer. Meanwhile, the role of shlinc-ITGB1 on cell migration was further confirmed by Tranwell assay. As shown in Figure 4A and B, the number of cells that migrated to the lower compartment was strongly reduced in the shlinc-ITGB1 group (69.067 ± 6.3), as compared to control (137.133 ± 7.159), indicating that linc-ITGB1 could play an essential role in GBC cell migration. Additionally, the amount of crystal violet in both groups reaffirmed our suggestion (Figure 4C). In addition, the impact of linc-ITGB1 knockdown on GBC-SD cell migration was visible, as determined by scratch and Transwell assays (Figure S1B, C and S2). These results strongly indicated that linc-ITGB1 could be a key regulator of GBC cell motility and migration.

**Linc-ITGB1 knockdown inhibited gallbladder cancer cell invasion**

Meanwhile, we examined the invasive potential of GBC cells infected with shCon and shlinc-ITGB1 by Matrigel invasion assay. GBC-SD/M cells were embedded in Matrigel, and the invasive growth of both groups was monitored over a period of 16 h. As shown in Figure 5A,B, the number of invaded cells was significantly decreased in the shlinc-ITGB1 group (69.6 ± 10.3), as compared to control (193.1 ± 8.3). Also, the amount of crystal violet in the shlinc-ITGB1 group was reduced by nearly threefold (Figure 5C). Taken together, these results indicated that knockdown of linc-ITGB1 by RNAi remarkably alleviated GBC cell migration and invasion, which are key contributors in GBC metastasis.

**Linc-ITGB1 knockdown reversed epithelial-to-mesenchymal transition**

We next sought to determine the underlying molecular mechanism by which linc-ITGB1 regulated GBC metastasis. EMT is critical manifestation of epithelial cell plasticity, which involves the dissolution of tight junctions, regulation of adherent junctions, reorganization of the actin cytoskeleton, loss of epithelial polarity, and acquisition of mesenchymal cell polarity (26,27). It has been shown to play a critical role in increasing the aggressiveness of GBC (3). The expression of EMT markers, such as E-cadherin, β-catenin, and vimentin were thereby analyzed by qRT-PCR. A trend for increased β-catenin expression but decreased vimentin expression was observed in GBC-SD/M cells after linc-ITGB1 knockdown (Figure 6A). Furthermore, Western blot confirmed that knockdown of linc-ITGB1 downregulated vimentin, slug, and TCF8 expression and upregulated β-catenin expression at protein levels (Figure 6B). These results suggested that linc-ITGB1 potentially promoted GBC invasion and metastasis by accelerating the process of EMT.

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**Figure 2:** Effect of linc-ITGB1 knockdown on cell proliferation in gallbladder cancer (GBC)-SD/M cells. The proliferative rate was much lower in GBC-SD/M cells infected with shlinc-ITGB1 than in shCon-infected cells, as determined by MTT assay. ***p < 0.001, compared to shCon.

**Figure 3:** Effect of linc-ITGB1 knockdown on wound healing in gallbladder cancer (GBC)-SD/M cells. (A) Representative images of uncovered wound areas in GBC-SD/M cells after shlinc-ITGB1 or shCon treatment for 36 h. Scale bar: 250 μm. (B) Relative migration areas of GBC-SD/M cells infected with shlinc-ITGB1 or shCon during the gap closure were measured with Image-Pro Plus. **p < 0.01, compared to shCon.
Discussion

Recently, an increasing number of studies have revealed that lincRNAs, a group of lncRNAs located within the intergenic regions of certain genes, are playing critical roles in multiple stages of cancer progression. For instance, the expression of a lincRNA encoded by a gene located next to POU3F3 (linc-POU3F3) was remarkably higher in esophageal squamous cell carcinoma (ESCC) tissues than neighboring non-tumor tissues and in vitro analysis revealed that linc-POU3F3 overexpression increased ESCC cell proliferation and colony formation ability (23). The expression levels of lincRNA-p21 in patients with stage I colorectal cancer (CRC) were significantly lower than those with stage III tumors, suggesting that lincRNA-p21 may contribute to CRC progression (28). In all, these findings indicate that lincRNAs might be prospective therapeutic targets in cancer therapy. Recently, advances in

Figure 4: Effect of linc-ITGB1 knockdown on cell migration in gallbladder cancer (GBC)-SD/M cells. The migration of GBC-SD/M cells was determined using the transwell chambers. (A) Cells migrating to the lower chamber was stained by crystal violet and photographed. Scale bar: 100 μm. (B) The number of migrated cells and (C) the amount of crystal violet in both groups were counted and analyzed. ***p < 0.001, compared to shCon.

Figure 5: Effect of linc-ITGB1 knockdown on invasion in gallbladder cancer (GBC)-SD/M cells. The invasive ability of GBC-SD/M cells was determined using the matrigel invasion chambers. (A) Cells invading to the lower chamber were stained by crystal violet and photographed. Scale bar: 100 μm. (B) The number of invaded cells and (C) the amount of crystal violet in both groups were counted and analyzed. *p < 0.05, ***p < 0.001, compared to shCon.
biological drugs, led by nucleic acid drugs (i.e., antisense oligonucleotides and siRNAs), have been reviewed by researchers, suggesting directions for the development of lincRNA-targeting treatment modalities (29). Notably, lentivirus-mediated RNAi system has also shown great potentials in molecular targeting therapy in various cancers, including pancreatic cancers, glioblastoma, breast cancers, and so on (30–33). In the present study, we identified a new lincRNA, linc-ITGB1, in a GBC cell line with higher metastatic potential. Furthermore, we employed lentivirus-mediated RNAi to silence linc-ITGB1 expression in GBC-SD/M cells and found that linc-ITGB1 knockdown could remarkably suppress GBC-SD/M cell proliferation, migration, and invasion in vitro.

Khalil et al. (34) analyzed chromatin-state maps of several cell types and examined whether many lincRNAs are physically associated with polycomb repressive complex (PRC)2. Interestingly, approximately 20% of lincRNAs are bound by PRC2, and some other lincRNAs are associated with other complexes that modify chromatin structure. This indicates that lincRNAs may regulate downstream gene expression by interacting with chromatin-modifying complexes. An extensively studied lincRNA, Hox transcript antisense intergenic RNA (HOTAIR), was found to be aberrantly expressed in several cancers and participate in cancer cell proliferation, EMT, and invasion (15,35). In this study, we found that linc-ITGB1 could contribute to GBC invasion via regulation of EMT. Losses of E-cadherin and membranous β-catenin are hallmarks of EMT, defined as the loss of epithelial differentiation and gain of mesenchymal phenotype, that is associated with acquisition of aggressive traits by cancer cells, invasion and metastasis (36–38). Vimentin is an intermediate filament that is used as a marker of mesenchymal cells to distinguish them from epithelial cells (39). Increased vimentin expression is frequently used as an EMT marker in cancer. Slug (also known as SNAI2), a member of the Snail family of transcriptional repressors, is capable of repressing E-cadherin expression and thereby triggering EMT (40,41), suggesting that it may act as an invasion promoter. TCF8 (ZEB1) is also an E-cadherin transcriptional repressor (42). As expected, knockdown of linc-ITGB1 resulted in a significant increase in β-catenin expression and a concomitant decrease in vimentin, slug, and TCF8 expression, which indicated that EMT could be involved in linc-ITGB1-induced GBC metastasis. However, we need to do further research to prove that there is a direct correlation between the accelerating the EMT process and linc-ITGB1 promoting the invasion and metastasis of gallbladder cancer.

Together, our findings indicate that lincRNA-ITGB1 is an important regulator of GBC metastasis and may serve as a potential therapeutic target for the treatment of GBC. Further investigation is needed to examine the relationship between lincRNA-ITGB1 expression and clinicopathological features in GBC cases.

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Author Contributions

PD designed the study and analyzed the data. LW and YJZ conducted the experiments and wrote the initial draft of the manuscript. WJL and JHL performed the experiments. JSM and YBL contributed to the interpretation of the data and made editorial comments on the manuscript.

Conflict of Interest

None.

References


Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Effect of linc-ITGB1 knockdown on wound healing in GBC-SD cells.

Fig. S2 Effect of linc-ITGB1 knockdown on cell migration in GBC-SD cells. The migration of GBC-SD cells was determined using the transwell chambers.