## *Original Article*
# Lnc-LFAR1 affects intrahepatic cholangiocarcinoma proliferation, invasion, and EMT by regulating the TGFβ/Smad signaling pathway

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**Abstract:** Intrahepatic cholangiocarcinoma (ICC) has had an increasing incidence in recent years. It exhibits high recurrence and metastasis rates, so it is a global health problem. LncRNAs have long been thought to have a role in regulating tumorigenesis and tumor progression and in playing a carcinogenic or tumor suppressor role in malignant cells. Lnc-LFAR1 is enriched in liver tissue, but its role and mechanism in ICC have not been elucidated. ICC cell line QBC939 cells were randomly divided into 3 groups, including a control group, an lnc-LFAR1 overexpression group, and an lnc-LFAR1 siRNA group, the cells of which were transfected with pcDNA3.1-LFAR1 plasmid and lnc-LFAR1 siRNA, respectively. Real-time PCR was used to quantify lnc-LFAR1 expression. A tetrazolium salt (MTT) colorimetric assay was adopted to assess cell viability. A cell scratch assay was selected to assess cell migration. A transwell chamber assay was used to test cell invasion. E-cadherin and vimentin expressions were detected using Western blot. The TGFβ/Smad signaling pathway was measured using real-time PCR. The transfection of pcDNA3.1-LFAR1 significantly increased the expression of lnc-LFAR1, promoted cell proliferation, facilitated cell migration and invasion, reduced E-cadherin levels, enhanced vimentin expression, upregulated TGF-β1 and Smad2 and Smad4 expressions ($P < 0.05$). Lnc-LFAR1 siRNA transfection clearly downregulated lnc-LFAR1 expression, inhibited cell proliferation, suppressed cell migration and invasion, increased E-cadherin expression, and decreased vimentin, TGF-β1, Smad2, and Smad4 levels ($P < 0.05$). Targeting lnc-LFAR1 can inhibit cell proliferation, migration, invasion, and EMT by regulating the TGFβ/Smad pathway to affect the occurrence and development of cholangiocarcinoma.

**Keywords:** Cholangiocarcinoma, lnc-LFAR1, TGFβ/Smad signaling pathway, proliferation, invasion, EMT

## Introduction

Intrahepatic cholangiocarcinoma (ICC) is the most common malignant tumor in the intrahepatic biliary epithelium. Its incidence is second only to hepatocellular carcinoma in primary liver malignancy, with an increasing trend [1, 2]. The onset of ICC is concealed, with no obvious symptoms or signs in the early stages. Most patients are not found in time, leading to rapid development and a high mortality rate, which seriously threatens global health [3, 4]. Adenocarcinoma is the main pathological type of ICC, which can easily invade blood vessels and lymphatic vessels, resulting in high recurrence and metastasis rates [5]. There are many factors involved in the pathogenesis of ICC, including viral infections, genetic factors, and physical and chemical factors [6]. At present,

surgical resection or liver transplantation is the main treatment method for ICC, but the prognosis of patients with surgical resections is poor, and the availability of livers for liver transplantation is rare. The long-term survival rate is still low, which causes great pain and economic burden to patients and their families [7, 8]. Understanding the mechanism of ICC is a difficult medical challenge. At present, the molecular markers of ICC have not been elucidated, so there is no effective molecular target [9]. The elucidation of the molecular markers and molecular targets of ICC is conducive to its early prevention and treatment.

The protein encoding genes encode fewer than 2% of the human genome. Most transcripts are composed of non-coding RNAs. LncRNAs, transcripts over 200 nt in length, are not involved in

encoding proteins that are abundant in the genome [10, 11]. Despite their poor conservation, lncRNAs can be regulated by transcription factors with tissue or cell specificity. They are involved in cell differentiation and somatic regulation [12, 13]. Recent studies found that lncRNAs regulate gene expression through epigenetic and transcriptional methods, and thus participate in various biological processes such as cell proliferation, survival, and differentiation [14, 15]. Lnc-LFAR1 is a newly discovered lncRNA with a 734 nt transcript containing only one exon and is enriched in liver tissue [16]. However, its role and mechanism in ICC has not been elucidated. Therefore, this study aimed to investigate the role and possible mechanism of lnc-LFAR1 in the biological behavior of ICC cell QBC939.

## Materials and methods

### Main reagents and instruments

The ICC QBC939 cell line was preserved in the Hunan University laboratory. DMEM medium, fetal bovine serum (FBS), and penicillin-streptomycin were purchased from Hyclone. Dimethyl sulfoxide (DMSO) and MTT powder was purchased from Gibco. Trypsin-EDTA digest was purchased from Sigma. PVDF membrane was purchased from Pall Life Sciences. Western blot related chemical reagent was purchased from Beyotime. ECL reagent was purchased from Amersham Biosciences. Rabbit anti-human E-cadherin monoclonal antibody, rabbit anti-human vimentin monoclonal antibody, and goat anti-rabbit horseradish peroxidase (HRP) labeled IgG secondary antibody were purchased from Cell Signaling. An RNA extraction kit, a reverse transcription kit, and lipo2000 reagent were purchased from Invitrogen. The pcDNA3.1-LFAR1 plasmid and lnc-LFAR1 siRNA were synthesized by Genepharma. Transwell chambers were purchased from Hyclone. The Labsystem Version 1.3.1 microplate reader was purchased from the Bio-rad Corporation. The ABI 7700 Fast Quantitative PCR Reactor was purchased from ABI. Other commonly used reagents were purchased from Sangon.

### Methods

*QBC939 cell culture and grouping:* The QBC939 cells preserved in liquid nitrogen were water bathed at 37°C until the cells were completely thawed. The cells were cultured at 37°C and 5% $CO_2$ for 24-48 h. Next, the QBC939 cells were seeded in a culture dish at a density of 1 × 10$^7$ cells/cm$^2$ with complete medium including 10% FBS, 90% high glucose DMEM medium, 100 U/ml penicillin, and 100 μg/ml streptomycin. QBC939 cells were cultured in vitro and randomly divided into 3 groups, including a control group, an lnc-LFAR1 overexpression, and an lnc-LFAR1 siRNA group, the cells of which were transfected with pcDNA3.1-LFAR1 plasmid and lnc-LFAR1 siRNA, respectively.

*pcDNA3.1-LFAR1 and lnc-LFAR1 siRNA transfection:* The pcDNA3.1-LFAR1 plasmid and lnc-LFAR1 siRNA were transfected into QBC939 cells, respectively. The plasmid sequence of pcDNA3.1-LFAR1 is 5'-TAAACCATAGAGCAATCA-TA-3'. The lnc-LFAR1 siRNA sequence is 5'-AUCGUGUCAGGUCUGAGA-3'. pcDNA3.1-LFAR1, and the lnc-LFAR1 siRNA liposomes were separately added to 200 μl of serum-free DMEM medium and incubated at room temperature for 15 min. The mixed lipo2000 was separately mixed and incubated at room temperature for 30 min. The mixture was added to the cells in a 6-well plate together with 1.6 ml of serum-free DMEM medium and cultured in a 5% $CO_2$ incubator at 37°C for 6 hours. The medium was replaced and the cells were further cultured for 48 hours.

*Real-time PCR:* Total RNA was extracted from the cells by Trizol and reverse transcribed to cDNA. The primers were designed by PrimerPremier 6.0 software and synthetized by Invitrogen (**Table 1**). Real-time PCR was performed at 55°C for 1 min, followed by 35 cycles of 92°C for 30 s, 58°C for 45 s, and 72°C for 35 s. GAPDH was selected as an internal reference. The relative expressions of the mRNA were calculated using the $2^{-\Delta Ct}$ method.

*MTT assay:* The QBC939 cells in the logarithmic phase were seeded in a 96-well plate at 3000 cells/well, and 20 μl MTT was added for 4 h. Next, 150 μl DMSO was added to the plate for 10 min and tested at 570 nm to obtain the absorbance value (A).

*Transwell assay:* The Transwell chamber was coated with 50 mg/L Matrigel diluted at 1:5 and air-dried at 4°C. 100 μl of tumor cell suspension prepared by serum-free medium and 10% FBS DMEM medium were added to the inner and outer chambers with 3 replicate wells in each group. Then the chamber was placed in

**Table 1.** Primer sequences

| Gene | Forward 5'-3' | Reverse 5'-3' |
|------|---------------|---------------|
| GADPH | AGTCTGACCAGTTTGCTGG | TAACCCGTAGTCAGATGTGGT |
| lnc-LFAR1 | TCCTAGACTCCCAGTTTCT | GTGTATAGTAGTGATGGT |
| TGF-β1 | CTAGACTCCTAACCTATAG | ATAGTTGATCTCAGTATA |
| Smad2 | TAGATAATCACCGATCATC | ACAGTAGACCAACAGT |
| Smad4 | CTCAATTCATCCACTCGAAG | GCACAGTGCTCGATGCTT |
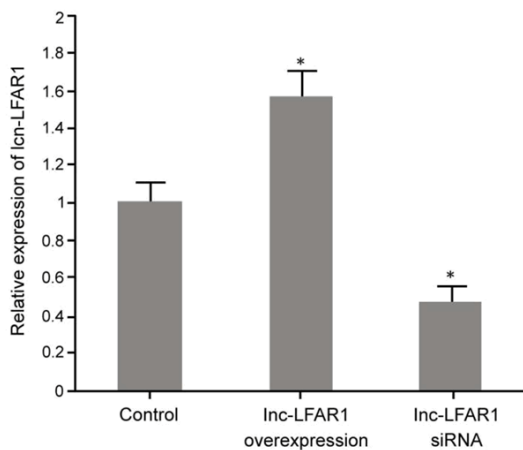


**Figure 1.** Lnc-LFAR1 expression in ICC QBC939 cells. *P < 0.05, compared with the control.
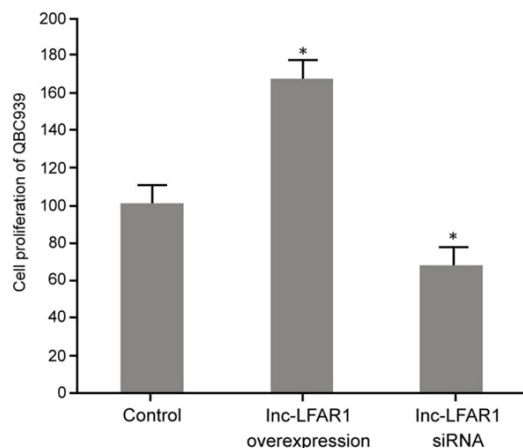


**Figure 2.** The influence of regulating lnc-LFAR1 on QBC939 proliferation. *P < 0.05, compared with control.

a 24-well plate. After 48 hours of culture, the Transwell chamber was washed with PBS, and the cells above the membrane were removed. Next, the membrane was fixed in ice ethanol. After staining with crystal violet, the cells in the lower layer of the microporous membrane were counted. The experiment was repeated three times.

*Cell scratch assay:* 5 × 10$^5$ cells were passaged and inoculated into a 6-well plate. A marker pen was placed behind the 6-well plate to evenly draw a horizontal line, which was passed through the hole every 0.5 to 1 cm. At least 5 lines were drawn through each hole. After 24 hours, the tip was used to draw a line as far as possible. The cells were washed 3 times with PBS and cultured in serum-free DMEM medium at 37°C and 5% CO$_2$. The results were observed after 48 hours.

*Western blot*

The cells were lysed on ice and quantified using the BCA method. The isolated proteins were electrophoresed using 10% SDS-PAGE. The gel was transferred to PVDF membrane using the semi-dry transfer method at 100 mA for 2 h. After being blocked by 5% skim milk for 2 h, the membrane was incubated in E-cadherin and vimentin primary antibodies at 4°C overnight. After being incubated in a secondary antibody in the dark for 30 min, the membrane was imaged using a chemiluminescence reagent for 1 min and analyzed by image processing system software and Quantity One software. The experiment was repeated four times (n = 4).

*Statistical analysis*

All data analyses were performed using SPSS 16.0 software. The measurement data were presented as the mean ± standard deviation and compared using one-way ANOVA. P < 0.05 was considered statistically significant.

**Results**

*Lnc-LFAR1 expression in QBC939 cells*

Real-time PCR was used to test lnc-LFAR1 expression in ICC QBC939 cells. pcDNA3.1-LFAR1 plasmid transfection significantly promoted the expression of lnc-LFAR1 compared with the control group (P < 0.05). Lnc-LFAR1 siRNA transfection obviously downregulated the expression of lnc-LFAR1 compared with the control group (P < 0.05) (**Figure 1**).

*The impact of lnc-LFAR1 on QBC939 cell proliferation*

The effect of pcDNA3.1-LFAR1 plasmid and lnc-LFAR1 siRNA transfection on QBC939 cell pro-
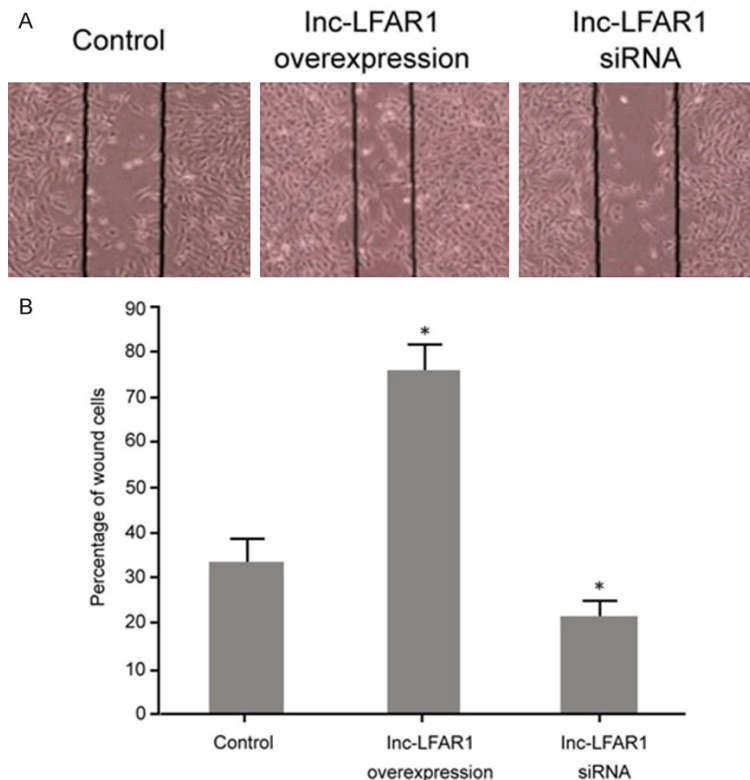
Figure 3. The influcence of lnc-LFAR1 on QBC939 cell migration. A. Cell scratch assay detection of the impact of regulating lnc-LFAR1 on QBC939 cell migration. B. Migration ability analysis. *$P < 0.05$, compared with control.
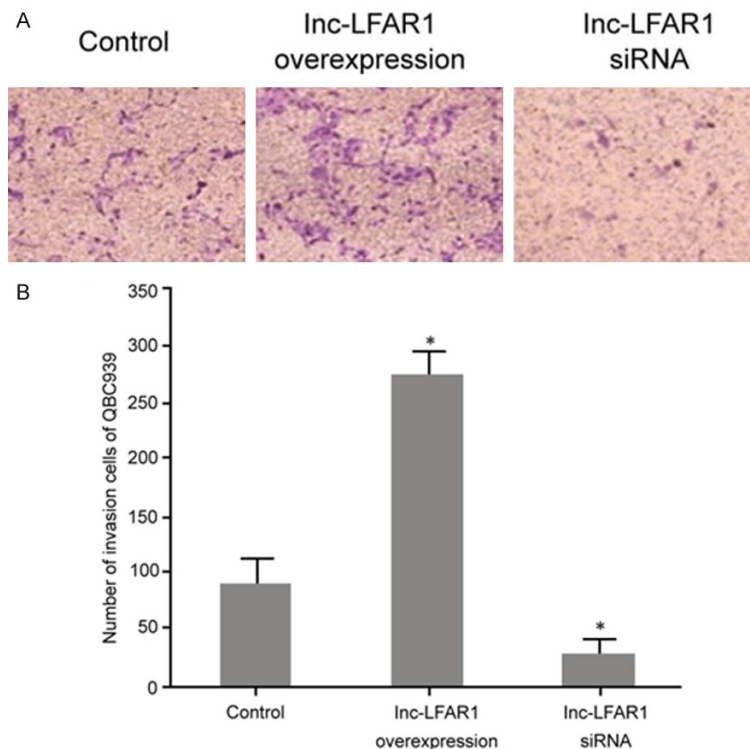


**Figure 4.** The effect of lnc-LFAR1 on QBC939 cell invasion. A. Transwell assay detection of the impact of regulating lnc-LFAR1 on QBC939 cell invasion. B. Migration ability analysis. *$P < 0.05$, compared with control.

liferation was determined using an MTT assay. It showed that the upregulation of lnc-LFAR1 expression by the pcDNA3.1-LFAR1 plasmid markedly promoted the proliferation of KB cells compared with the control group ($P < 0.05$). A decreased expression of lnc-LFAR1 markedly inhibited QBC939 cell proliferation compared with the control group ($P < 0.05$) (**Figure 2**).
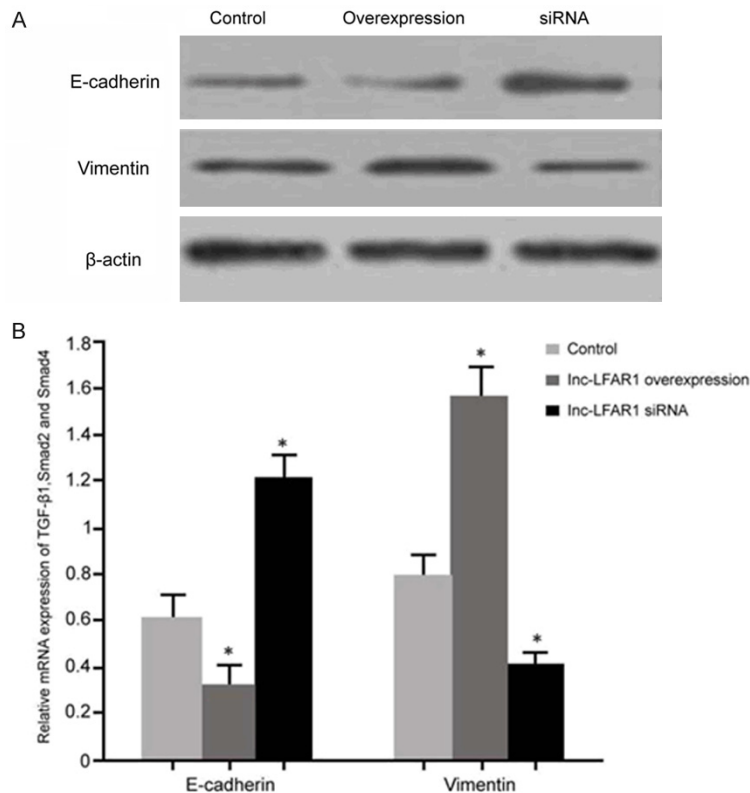
*The influence of lnc-LFAR1 on QBC939 cell migration*

A cell scratch assay was used to determine the effect of the pcDNA3.1-LFAR1 plasmid and lnc-LFAR1 siRNA transfection on the migration ability of QBC939 cells. It was demonstrated that the upregulation of lnc-LFAR1 expression markedly promoted the migration of QBC939 cells compared with the control group ($P < 0.05$). The transfection of lnc-LFAR1 siRNA apparently decreased the lnc-LFAR1 expression and inhibited QBC939 cell migration compared with the control group ($P < 0.05$) (**Figure 3**).
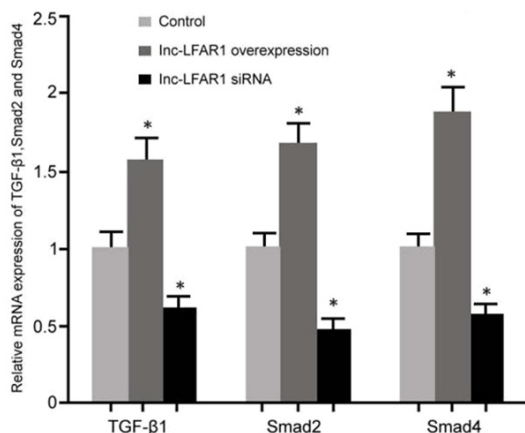
*The effect of lnc-LFAR1 on QBC939 cell invasion*

The Transwell assay was selected to determine the effect of the pcDNA3.1-LFAR1 plasmid and lnc-LFAR1 siRNA on the invasion of QBC939 cells. It was revealed that the upregulation of the lnc-LFAR1 expression after pcDNA3.1-LFAR1 plasmid transfection sig-

Figure 5. The impact of lnc-LFAR1 on EMT in QBC939 cells. A. Western blot detection of EMT protein expression; B. E-cadherin and vimentin protein expression analyses. *$P < 0.05$, compared with the control.



Figure 6. The influence of lnc-LFAR1 on the TGFβ/Smad signaling pathway in the QBC939 cells. *$P < 0.05$, compared with the control.

nificantly enhanced KB cell invasion compared with the control group ($P < 0.05$). Transfection of lnc-LFAR1 siRNA clearly decreased the expression of lnc-LFAR1 and restrained QBC939 cell invasion compared with the control group ($P < 0.05$) (**Figure 4**).

## The impact of lnc-LFAR1 on EMT in QBC939 cells

The effects of transfection of the pcDNA3.1-LFAR1 plasmid and the lnc-LFAR1 siRNA on the EMT of QBC939 cells were detected by Western blot. It was shown that the upregulation of lnc-LFAR1 expression after transfection of pcDNA3.1-LFAR1 plasmid significantly decreased E-cadherin and vimentin expressions compared with the control group ($P < 0.05$). The decreased expression of lnc-LFAR1 clearly enhanced the E-cadherin and vimentin levels compared with the control ($P < 0.05$) (**Figure 5**).

## The influence of lnc-LFAR1 on the TGFβ/Smad signaling pathway in QBC939 cells

The effects of the pcDNA3.1-LFAR1 plasmid and of lnc-LFAR1 siRNA transfection on the TGFβ/Smad signaling pathway in the QBC939 cells was analyzed by real-time PCR. It was revealed that the upregulation of lnc-LFAR1 expression after the transfection of pcDNA3.1-LFAR1 markedly upregulated TGF-β1, Smad2, and Smad4 levels compared with the control group ($P < 0.05$). A decreased expression of lnc-LFAR1 apparently inhibited TGF-β1, Smad2, and Smad4 expressions compared with the control ($P < 0.05$) (**Figure 6**).

## Discussion

As a tumor with a high incidence in hepatobiliary surgery, ICC is highly difficult to diagnose and treat. At present, no molecular markers or treatments have been found. Surgery is the only treatment method, and chemotherapy is not effective in ICC patients in the advanced stages [17]. As a transcriptional and post-transcriptional regulator, lncRNA has potential as a therapeutic target and can be used as an indicator of tumor prognosis [18, 19]. It was found that lnc-LFAR1 has a short open reading frame (ORF) that plays an important role in translation initiation [20]. In liver fibrosis, lnc-LFAR1 is in-

volved in disease progression by regulating the TGFβ/Smad signaling pathway [16]. However, the role and mechanism of lnc-LFAR1 in ICC has not been elucidated. Our results confirmed that the transfection of the pcDNA3.1-LFAR1 plasmid overexpressed the lnc-LFAR1 level, promoted cell proliferation, and enhanced the cell migration and invasion abilities in ICC cells. Conversely, the transfection of lnc-LFAR1 siRNA significantly reduced lnc-LFAR1 expression, restrained cell proliferation, and attenuated cell migration and invasion. This suggests that the up-regulation of lnc-LFAR1 can promote the development and progression of intrahepatic cholangiocarcinoma, and the down-regulation of lnc-LFAR1 can inhibit its proliferation, invasion, and migration.

The epithelial-mesenchymal transition (EMT) refers to epithelial cells transforming into mesenchymal cells during tumor invasion and metastasis [21]. EMT is accompanied by decreased E-cadherin expression and upregulated vimentin levels [22, 23]. This study further aimed to analyze the regulation of lnc-LFAR1 on EMT in intrahepatic cholangiocarcinoma. The up-regulation of lnc-LFAR1 inhibited E-cadherin expression and facilitated vimentin levels in intrahepatic cholangiocarcinoma. However, the down-regulation of lnc-LFAR1 suppressed the expression of vimentin and enhanced the expression of E-cadherin in intrahepatic cholangiocarcinoma. TGF-β1, an important biological factor, can bind to its receptor to regulate the expression of downstream signaling factors and activate intracellular transcription factors. The TGF-β1 receptor is Smad, which can be associated with Smad2 and Smad4 to form a heteromeric complex. The activated Smad factor enters the nucleus and regulates the transcription of target genes to play an important role in cell biological behavior [24, 25]. This study confirmed that the overexpression of lnc-LFAR1 promotes the expression of TGF-β1, Smad2, and Smad4 in ICC cells, thereby upregulating the TGFβ/Smad signaling pathway. Inhibiting the expression of lnc-LFAR1 suppresses the expression of TGF-β1, Smad2 and Smad4 and further down-regulates the TGFβ/Smad signaling pathway, suggesting that it can regulate ICC proliferation, invasion, and EMT. Further research, should deeply analyze the expression of lnc-LFAR1 in the tumor tissues of ICC patients and its correlation with their clinicopathological features, thus providing a reference for a clinical therapeutic target.

## Conclusion

Targeting lnc-LFAR1 can inhibit cell proliferation, migration, invasion, and EMT by regulating the TGFβ/Smad pathway to affect the occurrence and development of cholangiocarcinoma.

## Acknowledgements

## Disclosure of conflict of interest

None.

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