

Original Article

# LncRNA LIMT (LINC01089) contributes to sorafenib chemoresistance via regulation of miR-665 and epithelial to mesenchymal transition in hepatocellular carcinoma cells

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## Abstract

Hepatocellular carcinoma (HCC) is one of the most malignant tumors worldwide and HCC patients often develop drug resistance. Long non-coding RNAs (lncRNAs) are closely related to cell cycle, growth, development, differentiation, and apoptosis. Abnormally expressed lncRNAs have been proved to mediate drug resistance in tumor cells. However, the effect of LIMT on drug resistance has not been explored in HCC. In this study, we explored the effect of long non-coding RNA LIMT on drug resistance and its underlying mechanism in hepatocellular carcinoma (HCC). Our results showed that lncRNA LINC01089 (LIMT) expression is downregulated in 78.57% (44/56) of 56 HCC tumor tissue samples. LIMT expression is also downregulated in HCC cells compared with that in normal liver LO2 cells. Inhibition of LIMT increases the resistance to sorafenib and promotes cell invasion via regulation of epithelial to mesenchymal transition (EMT) in HCC. StarBase V3.0 was used to predict the potential binding site of miR-665 in *LIMT*. Furthermore, miR-665 participates in sorafenib resistance and also regulates the level of EMT-related proteins in HCC cells. A rescue experiment demonstrated that silencing of *LIMT* eliminates the inhibitory effect of the miR-665 inhibitor on sorafenib resistance in HCC cells. Taken together, our findings revealed that downregulation of LIMT increases the resistance of HCC to sorafenib via miR-665 and EMT. Therefore, LIMT, which serves as a therapeutically effective target, will provide new hope for the treatment of HCC.

**Key words** lncRNA LIMT, sorafenib, chemoresistance, EMT, HCC

## Introduction

Hepatocellular carcinoma (HCC) is one of the most pernicious tumors worldwide [1]. Because of the high rate of recurrence and metastasis, more and more patients are diagnosed with HCC every year and also with a poor prognosis [2]. Sorafenib, which acts as a multiple kinase inhibitor, is the first-line drug for the treatment of HCC in many countries [3]. Although sorafenib can extend HCC patients' survival time, its curative effect is short because of the development of drug-resistant cells. Some patients with HCC are not

only resistant to the long-term effects of sorafenib, but also show resistance in the early stage [4]. Thus, it is urgent to reveal the detailed mechanisms of sorafenib resistance in HCC.

Long non-coding RNAs (lncRNAs > 200 nucleotides) are closely related to a series of physiological activities, such as cell cycle, growth, development, differentiation, and apoptosis. Several abnormally expressed lncRNAs have been proved to mediate drug resistance in tumor cells. For example, inhibition of *HOTAIR* could decrease drug resistance in non-small-cell lung cancer (NSCLC) by

regulating the expression of Unc-51 like autophagy activating kinase 1 (ULK1) [5]. Mechanistically, lncRNAs and microRNAs (miRNAs) can interact to mediate drug resistance and cell invasion in various cancer cells. For instance, silencing of *SNHG16* was shown to increase chemotherapy resistance to sorafenib in HCC by upregulating miR-140-5p [6]. In addition, drug resistance was inhibited following transfection with a small interfering RNA (siRNA) targeting FOXD2-AS1 via the miR-98-5p/cytoplasmic polyadenylation element binding protein 4 (CPEB4) axis and the regulation of cell proliferation and invasion [7]. LncRNA LINC01089 (LIMT) functions as a metastasis inhibitory lncRNA, which was first discovered to be inhibited by epidermal growth factor (EGF) in breast cancer, and is related to cell migration and metastasis in different cancers [8,9]. However, the effect of LIMT on drug resistance has not been explored so far in HCC.

The process of epithelial to mesenchymal transition (EMT) accompanied by epithelial cells is rendered into cells containing mesenchymal phenotypes via special steps [10,11]. EMT is related to a great diversity of biological processes, such as embryonic development, tissue reconstruction, chronic inflammation, cancer metastasis, and drug resistance [12,13]. Cancer cells generally acquire multi-drug resistance during EMT. Alternatively, EMT is closely associated with chemo-resistance to cisplatin and doxorubicin in HCC [14–16]. In addition, evidence shows that EMT and its accompanying molecules are the key determinants of resistance to sorafenib therapy in HCC. Therefore, analysis of the molecular mechanism underlying EMT might help to improve drug resistance to achieve an anti-tumor effect.

This study intended to reveal the involvement of LIMT in sorafenib resistance and the potential underlying mechanism. It provides important evidence that LIMT may be a novel therapeutic target and a biomarker to predict the effect of sorafenib therapy in HCC.

## Materials and Methods

### Cell culture and the HCC human tissues

The human HCC cells (Huh7, Hep3B, and SNU449) were purchased from ATCC (Manassas, USA) and cultured separately in Dulbecco's modified Eagle's medium (DMEM), minimal essential medium (MEM), and Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Carlsbad, USA) supplemented with 10% FBS (Sigma, St Louis, USA) under a suitable environment (5% CO<sub>2</sub> at 37°C). The tumor tissue samples of HCC patients were obtained from Tongde Hospital of Zhejiang Province. All patients provided written informed consent and the study protocol was approved by the Clinical Research Ethics Committee of Tongde Hospital of Zhejiang Province.

### Cell viability

Cell viability in different treatment groups was determined by Cell Counting Kit-8 (CCK-8) assay. HCC cell suspension (5 × 10<sup>3</sup> cells) was inoculated into 96-well plates (100 µl/well) and cultured at 37°C with 5% CO<sub>2</sub> until the cells adhered. Next, 10 µL of CCK-8 solution (Dojindo, Tokyo, Japan) was added into each well and incubated at 37°C for 2 h. Thereafter, the optical density (OD) value of each well was detected at 450 nm with a microplate reader.

### Transfection

LIMT siRNAs (LIMT siRNA1:LIMT siRNA2:LIMT siRNA3 = 1:1:1), miR-665 siRNA, miR-665 mimics, miR-665 inhibitor and their corresponding negative controls were designed and manufactured by

GenePharma (Shanghai, China). Transfections were performed using lipofectamine 2000 (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. The sequences are shown in Table 1.

### Western blot analysis

The concentrations of extracted protein from different treatment groups were measured by bicinchoninic acid (BCA) protein assay. Before separation by SDS-PAGE, equal amounts of protein (40 µg) were denatured by heating with loading buffer at 100°C. The separated proteins on the gel were electro-transferred onto polyvinylidene fluoride (PVDF) membranes. After being blocked with 5% nonfat dry milk in TBS-T buffer for 2 h at 37°C, the membranes were incubated with indicated primary antibodies, including anti-fibronectin 1 (FN-1) antibody, anti-E-cadherin antibody, anti-vimentin antibody and anti-GAPDH antibody (1:1000 dilution; Abcam, Cambridge, UK), overnight at 4°C. Then the membranes were washed and incubated with the corresponding HRP-conjugated secondary antibodies (1:2000 dilution; Cell Signaling Technology, Beverly, USA). The immunoreactive protein bands were visualized using an ECL kit (GE Healthcare, Piscataway, USA). GAPDH was used as the loading control.

### qRT-PCR analysis

Total RNA was extracted from HCC cells using Trizol reagent (Invitrogen) and then reverse transcribed to cDNA using a Reverse Transcription kit (TaKaRa, Tokyo, Japan). Utilizing SYBR Premix Ex Taq (TaKaRa), qRT-PCR was run and analyzed on the Applied Biosystems Real-time PCR System (Foster City, USA). *GAPDH* and *U6* were used as reference controls for the lncRNA, mRNA, and miRNA. The primers used were as follows: *LIMT* forward primer: 5'-CGAATGGACAATCTTCTCTCTGTC-3', reverse primer: 5'-GCTA-GAGGTTGAGGGCCTGAGT-3'; and *miR-665* forward primer: 5'-ACCAGGAGGCTGAGGC-3', reverse primer: 5'-GAACATGTCTGC GTATCTC-3'; *GAPDH* forward primer: 5'-CGGAGTCAACGGATT TGGTCGTAT-3' reverse primer: 5'-AGCCTTCTCCATGGTGGTGA AGAC-3'; and *U6* forward primer: 5'-GCTTCGGCAGCACATATA CTAATAAT-3', reverse primer: 5'-CGCTTACGAATTGCGTGTCT AT-3'.

### Dual-luciferase reporter assays

Reporter plasmids containing the LIMT-3' UTR, which contained the mutant (MUT) or wild-type (WT) binding sequence for miR-665, were constructed by GenePharma. The plasmids were then transiently co-transfected into cells with luciferase reporter vectors together with the miR-665-mimic, miR-665 inhibitor, or control using Lipofectamine 2000. Forty-eight hours after transfection, the re-

**Table 1.** The siRNA sequences used in this study

Name	Sequence
LIMT-homo-177	Sense: 5'-CCAUUCAUGUCAGCAGUUATT-3'; Antisense: 5'-UAACUGCUGACAUGAAUGGTT-3'
LIMT-homo-295	Sense: 5'-GCAGAACGUGAGGGUGUAATT-3' Antisense: 5'-UUACACCCUCACGUUCUGCTT-3'
LIMT-homo-888	Sense: 5'-GCUUCCAACCUCCAUUGCATT-3'; Antisense: 5'-UGCAAUGGAGGUUGGAAGCTT-3'
miR-665 mimic	Sense: 5'-ACCAGGAGGCGUGAGGCCCCU-3'; Antisense: 5'-GGCCUCAGCCUCCUGUUU-3'
miR-665 inhibitor	5'-AGGGCCUCAGCCUCCUGGU-3'

lative luciferase activity of the WT or MUT LIMT-3'-UTR was measured using a dual-luciferase reporter assay kit (Promega, Madison, USA).

### Ethynyl deoxyuridine (EdU) assay

Cell proliferation was determined using a Click-iT<sup>®</sup> EdU Imaging kit according to the instructions of the manufacturer (Invitrogen). Briefly, HCC cells were seeded in 96-well plates at a density of  $3 \times 10^3$  cells/well in growth media. The medium was replaced by serum-free medium to synchronize the cells. After 24 h of incubation, the serum-free medium was replaced by growth media with the indicated concentrations and cultured for 48 h. After incubation with 20  $\mu$ M EdU for 3 h, Cell nuclei were stained with Hoechst 33342 (Invitrogen) at a concentration of 5  $\mu$ g/mL for 30 min. The percentage of positive cells in five random fields of view per slide was determined under an inverted fluorescence microscope (Olympus, Tokyo, Japan) and expressed relative to that in the untreated control cells.

### Cell cycle and apoptosis analysis

Cell cycle and apoptosis analysis were performed by flow cytometry using Cell Cycle Assay kit-PI/RNase Staining and Annexin V/FITC Apoptosis Detection kit (Roche, Basel, Switzerland) according to manufacturer's instructions. Cell cycle analysis was carried out by Cell Cycle Assay Kit-PI/RNase Staining. Briefly, the cell density was adjusted to  $2 \times 10^6$  cells/mL, and 1 mL of cell suspension was added into a 1.5-mL microtube and centrifuged at 1000 *g* for 3 min. Then the supernatant was discarded and 1 mL of 70% ethanol ( $-20^\circ\text{C}$ ) was added to the cell pellet to disperse the cells by vortexing and standing at  $4^\circ\text{C}$  for 2 h. After centrifugation at 1000 *g* for 3 min, the ethanol was removed and 1 mL PBS buffer was added to wash the cells. After centrifugation at 1000 *g* for 3 min, the supernatant was discarded and 0.5 mL of Working Solution (500  $\mu$ L assay buffer, containing 25  $\mu$ L PI solution and 2.5  $\mu$ L RNase solution) was added. After vortexing and incubation for 30 min at  $37^\circ\text{C}$  in the dark, the mixture was further incubated for 30 min at  $4^\circ\text{C}$  in the dark, followed by vortexing and filtering through a nylon mesh to remove cell clumps in the sample. Finally, cell cycle was detected by flow cytometry.

Cell apoptosis analysis was carried out using Annexin V/FITC Apoptosis Detection kit. Briefly, HCC cells (treated as above) were harvested by trypsinization, rinsed with ice-cold phosphate PBS, and centrifuged to remove the supernatant. Then, the cells were resuspended in 100  $\mu$ L  $1 \times$  binding buffer and incubated with Annexin V-FITC for 15 min in the dark at room temperature. Finally, a flow cytometer was used to determine the number of apoptotic cells from B2 and B4 quadrants.

### Transwell assay

HCC cell suspension (100  $\mu$ L of  $2.5 \times 10^4$  cells/ $\mu$ L) was added into the upper Transwell chamber (Corning Costar, New York, USA) coated with Matrigel (BD, Franklin Lakes, USA). After 48 h of incubation, the membranes were fixed with methanol for 30 min and stained with 0.1% crystal violet for 20 min. Then the infiltrated cells were counted and photographed in five random fields under a microscope (Olympus) at a magnification of  $100 \times$ .

### Nude mouse xenograft model

Female BALB/c *nu/nu* mice (4–5 weeks old) were purchased from

Shanghai SLAC Laboratory Animal Co., Ltd (Shanghai, China). Huh7 cells were subcutaneously injected into the left hip of three mice. After the tumor was formed, a small section (1  $\text{mm}^3$ ) of tumor tissue was inoculated into the experimental group nude mice. After 10 days, the tumors had a diameter of 0.5 cm and reached a volume of  $\sim 50$ – $100 \text{ mm}^3$ . The mice were randomly divided into four groups ( $n=3$  per group): control, sorafenib group, LIMT plasmid group, and LIMT plasmid + sorafenib group. LIMT plasmid was injected intratumorally four times from day 0 to day 14, while sorafenib was injected into the tail vein once every two days for two weeks. Tumor volumes were recorded every two days and body weight was measured daily. The tumor volume ( $\text{mm}^3$ ) was calculated using the formula:  $\text{Volume} = (\text{length} \times \text{width}^2/2)$ . The mice were sacrificed humanely on day 15 after treatment, and the resected tumors were weighed and subject to subsequent analysis.

### TUNEL analysis

The apoptosis in paraffin-embedded mouse tissue sections (5-mm) was determined by an *In situ* cell death detection kit (Roche, Basel, Switzerland). The apoptotic cells were observed under a light microscope (Olympus). The assay was independently repeated three times. The positive rates were measured using IPP 6.0 software (Media Cybernetics, Bethesda, USA).

### Statistical analysis

GraphPad software 8 (GraphPad Inc., San Diego, USA) was used for calculating statistical comparisons. Data were shown as the mean  $\pm$  SD and analyzed by two-tailed Student's *t*-test. A value of  $P \leq 0.05$  was considered to be significantly different. StarBase 3 (<http://starbase.sysu.edu.cn/index.php>) was used to analyze the relationship between LIMT and miR-665.

## Results

### The level of LIMT is associated with sorafenib resistance

To verify the relationship between the expression level of LIMT and sorafenib resistance, qRT-PCR was performed to detect LIMT expression in 56 pairs of HCC tumor tissues and matched adjacent tissues. The results showed that LIMT expression was down-regulated in 78.57% (44/56) HCC tumor tissue (Figure 1 A,B). We also investigated the relationship between LIMT expression level and clinical characteristics in the 33 HCC cancer cases to determine whether LIMT expression is related to clinical features. The results showed that low expression of LIMT in 33 HCC patients was closely related to large tumor size ( $P=0.0270$ ), TNM stage ( $P=0.0173$ ), and HBsAg ( $P=0.0221$ ). However, LIMT expression level was not correlated with other parameters, such as patient age, sex, alpha-fetoprotein (AFP), and portal vein tumor thrombus (Table 2). Next, the expression of LIMT was examined in HCC cells and normal liver epithelial LO2 cells, which showed that LIMT expression was reduced in HCC cells (Figure 1C). Furthermore, we found that the SNU449 cell line, with the lowest LIMT expression, was most resistant to sorafenib. The  $\text{IC}_{50}$  values for sorafenib in HCC cells are presented in Figure 1D,E.

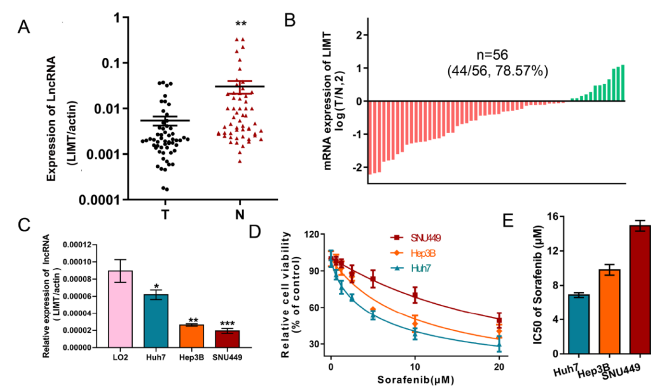
### Interference with LIMT enhances sorafenib resistance and promotes invasion by promoting EMT

To further reveal the chemoresistance effect of LIMT on sorafenib, HCC cells were transfected with LIMT siRNAs (LIMT siRNA1:LIMT siRNA2:LIMT siRNA3 = 1:1:1) and observed the changes of cell

**Table 2. Correlations between LIMT and clinical characteristics of 33 HCC cancer patients**

Clinical parameter		n = 33	High	Low	P-value
Sex	Female	9	6	3	0.1340
	Male	24	9	15	
Age (year)	< 60	19	10	9	0.5787
	≥60	14	6	8	
Stage	I-II	19	12	7	0.0173*
	III-IV	14	3	11	
Tumor size (cm <sup>3</sup> )	< 3	13	9	4	0.0270*
	≥3	20	6	14	
AFP	< 20	16	8	8	0.6109
	≥20	17	7	10	
Portal vein tumor thrombus	Yes	7	4	3	0.8764
	No	26	14	12	
HBsAg	Yes	16	4	12	0.0221*
	No	17	11	6	

AFP, alpha-fetoprotein.



**Figure 1. The level of LIMT is associated with sorafenib resistance** (A, B) LIMT expression was detected in 56 pairs of HCC tumor tissues and matched adjacent tissues. \*\* $P < 0.01$ . (C) LIMT expression was determined in HCC cells and normal liver LO2 cells. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ . (D,E) Cell viability was examined by CCK-8 assay after treatment with sorafenib, and the  $IC_{50}$  values are represented by a bar chart.

viability and proliferation. As shown in Figure 2, LIMT silencing increased the resistance to sorafenib by enhancing cell viability and proliferation, while LIMT plasmid treatment decreased this effect in HCC cells (Figure 2A,B and Supplementary Figure S1A). Transwell analysis was then used to determine the invasion ability of HCC cells. Compared with cells treated with sorafenib alone, the number of HCC cells that invaded the Matrigel layer was increased after transfection with LIMT siRNAs (Figure 2C). EMT was also found to play an important role in HCC resistance to sorafenib and cell invasion in a variety of cancers [17–19]. Western blot analysis of the EMT-related proteins, including FN-1, E-cadherin, and Vimentin, showed that LIMT silencing promoted the sorafenib-induced reduction in E-cadherin, whereas it upregulated sorafenib-induced increase in the expressions of FN-1, Vimentin and Twist 1 (Figure 2D). The phenotypic changes was shown in Supplementary Figure S3A. Furthermore, flow cytometry analysis showed

that sorafenib treatment could increase cell apoptosis and enhance the rate of G2/S. However, LIMT siRNA combined with sorafenib could reduce cell apoptosis and the rate of G2/S compared with those in the sorafenib group (Supplementary Figure S1B,C). The cell interference efficiency of LIMT was shown in Supplementary Figure S2A–C.

### LIMT negatively regulates miR-665 expression

To further explore the mechanisms underlying the effect of LIMT, the potential miRNAs binding site in LIMT was identified from multidimensional sequencing data using StarBase V3.0 and the reporter plasmids containing the LIMT-3'UTR were further constructed, which contained the mutant (MUT) or wild-type (WT) binding sequences to verify the interaction between LIMT and miR-665 by Dual-Luciferase Reporter Assays (Figure 3A,B). Moreover, we detected the levels of miR-665, E-cadherin and Vimentin in HCC tumor tissues and matched adjacent tissues, and the results revealed that E-cadherin was down-regulated, and the expressions of miR-665 and Vimentin were increased in HCC tumor tissues (Figure 3C), indicating that LIMT was negative-correlated with the level of miR-665 (Figure 3C), which is consistent with results from starBase v3.0 (Supplementary Figure S2D). The level of miR-665 was significantly increased after LIMT siRNA transfection, while its expression was downregulated after treatment with LIMT plasmid (Figure 3D). Transfection with miR-665 mimics reduced LIMT level, while the miR-665 inhibitor upregulated LIMT level (Figure 3E). These data suggest that LIMT level is inversely correlated with the miR-665 level in HCC cells.

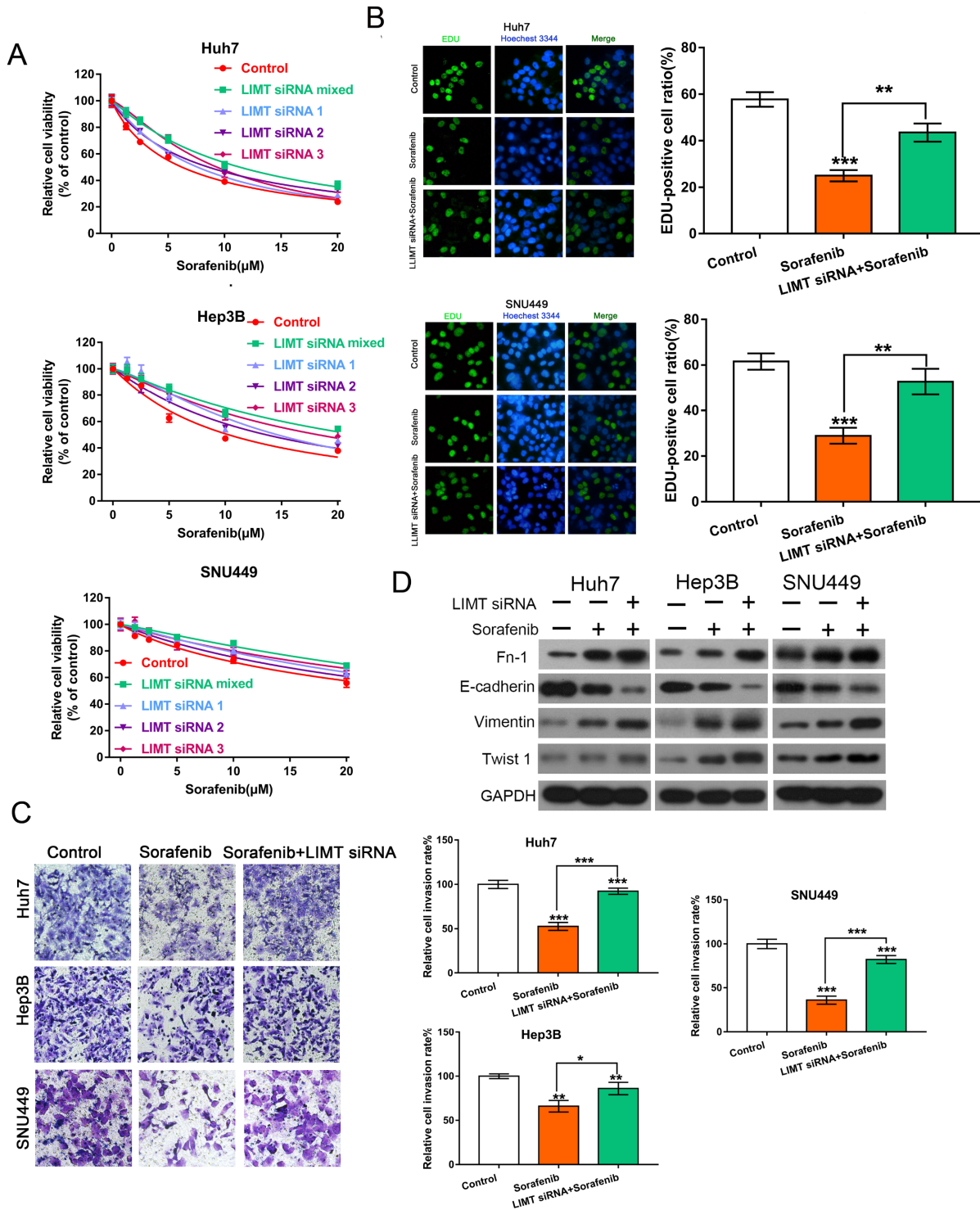
### miR-665 regulates resistance to sorafenib

To investigate the functions of miR-665 in HCC cells, we also examined resistance to sorafenib and cell invasion after treatment with miR-665 mimics, inhibitor, with or without sorafenib. The results showed that miR-665 mimics increased sorafenib resistance, whereas the miR-665 inhibitor enhanced sorafenib sensitivity (Figure 4A). EdU analysis also confirmed these results (Figure 4B). The number of HCC cells passing through the membrane was increased after miR-665 mimic treatment, indicating that miR-665 mimics could enhance the invasion capability of HCC cells (Figure 4C). To reveal whether miR-665 participates in the EMT process, we determined the levels of EMT-related proteins. Compared with the sorafenib group, miR-665 mimics combined with sorafenib could downregulate E-cadherin level and increase FN-1, Vimentin, Twist 1 expressions. However, miR-665 inhibitor plus sorafenib could reverse these effects (Figure 4D). The phenotypic changes are shown in Supplementary Figure S3B.

### LIMT regulates sorafenib resistance through regulating miR-665 and EMT

To verify whether LIMT plays the role in sorafenib resistance via the regulation of miR-665 and EMT, we transfected the miR-665 inhibitor into HCC cells along with the LIMT siRNAs, followed by treatment with sorafenib to perform a rescue experiment. As expected, the results showed that the LIMT siRNA inhibited the increase of miR-665 inhibitor-induced sorafenib sensitivity, and showed no significant difference between the control group and silencing of LIMT plus the miR-665 inhibitor group (Figure 5A–C). MiR-665 inhibitor combined with LIMT siRNA could reduce the invasion ability compared with LIMT siRNA treatment alone (Figure



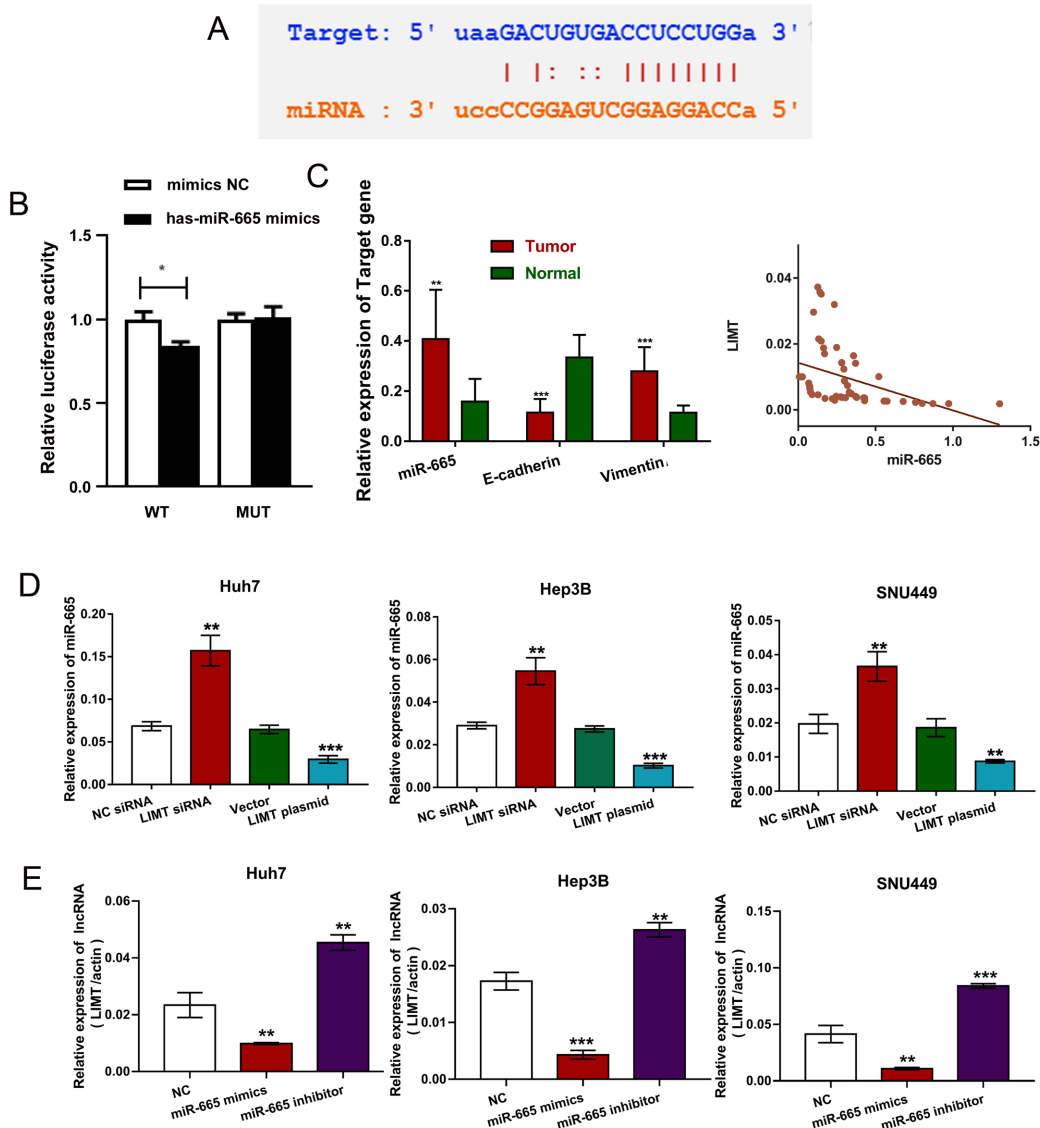


**Figure 2. Interference with LIMT enhances sorafenib resistance and promotes invasion via regulation of EMT** (A,B) CCK-8 and EdU determination of the cell viability and proliferation of HCC cells following transfection with LIMT siRNAs (LIMIT siRNA 1, LIMIT siRNA 2, LIMIT siRNA 3) and combined with or without sorafenib. \*\* $P < 0.01$  and \*\*\* $P < 0.001$ . (C) Transwell invasion analysis of HCC cells transfected with LIMT siRNA combined with sorafenib. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ . (D) Western blot analysis of FN-1, E-cadherin, Vimentin and Twist 1 expression levels in HCC cells.

5D). Western blot analysis also confirmed that miR-665 inhibitor combined with LIMT silencing could inhibit EMT compared with LIMT siRNA treatment alone (Figure 5E). These results indicated that inhibition of LIMT might enhance chemotherapy resistance to

sorafenib by upregulating the effect of miR-665.

**LIMIT over-expression suppresses tumor growth *in vivo***  
To explore the effect of LIMT on sorafenib resistance *in vivo*, tumor



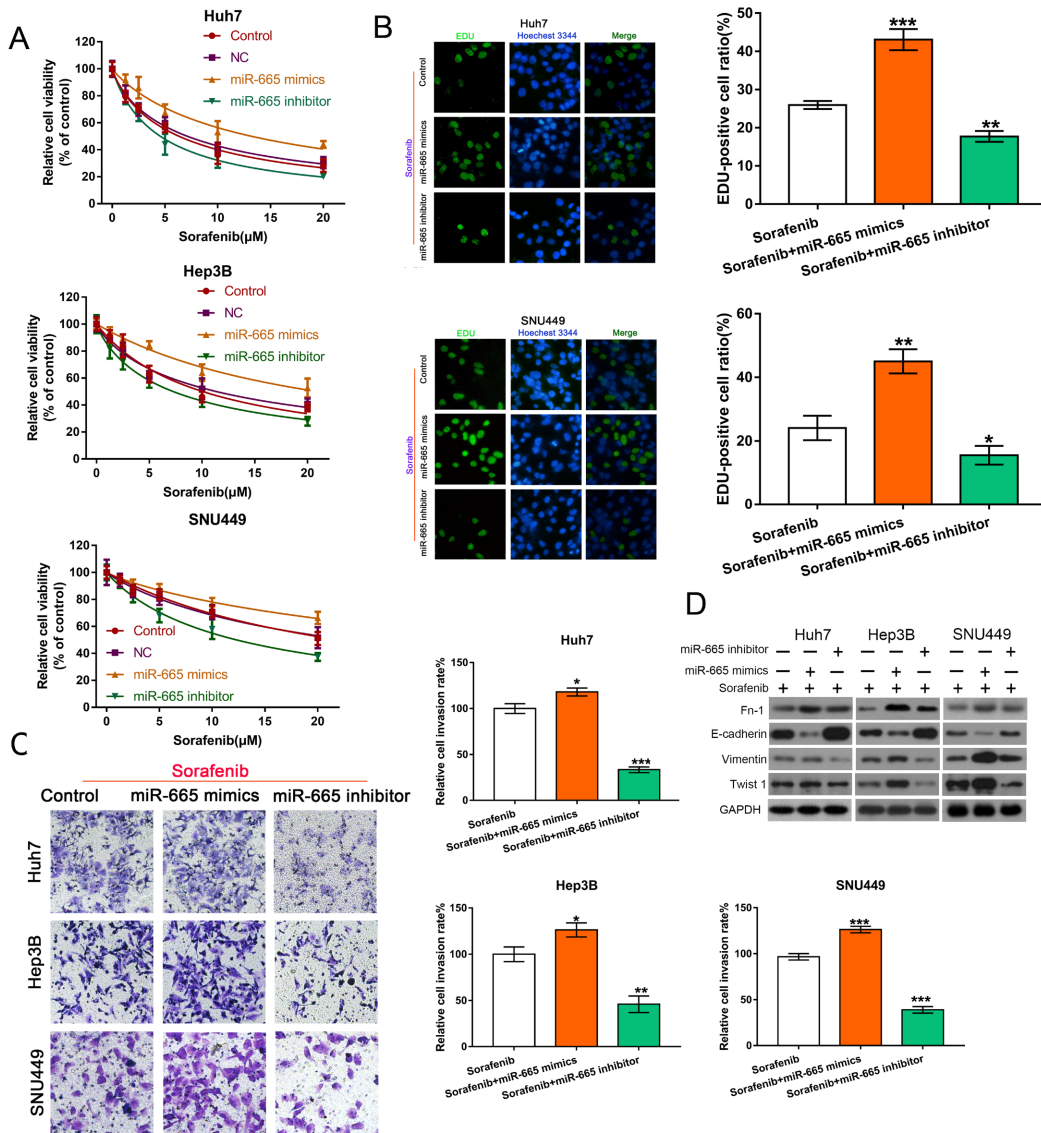
**Figure 3. LIMT is negatively correlated with miR-665 expression** (A) StarBase V3.0 was used to predict the potential binding site for miR-665 in LIMT. (B) Relative luciferase activity of HCC cells co-transfected with WT-LIMT or Mut-LIMT and NC, or miR-665 mimics.  $*P < 0.05$ . (C) The levels of miR-665 and EMT-related mRNAs were detected in HCC tumor tissues and matched adjacent tissues. The correlation between LIMT and miR-665 ( $**P < 0.01$  and  $***P < 0.001$ ). (D) The level of miR-665 in HCC cells treating with LIMT siRNA or the LIMT overexpressing plasmid was determined by qRT-PCR.  $**P < 0.01$  and  $***P < 0.001$ . (E) LIMT expression was detected after incubation with miR-665 mimics or inhibitors.  $**P < 0.01$  and  $***P < 0.001$ .

nude mouse model was established and treated with normal saline alone, sorafenib alone, LIMT over-expression alone, or sorafenib plus LIMT over-expression. The results showed that the tumor volume was significantly reduced in the sorafenib plus LIMT over-expression group (Figure 6A,B). TUNEL analysis revealed that sorafenib plus LIMT over-expression treatment could enhance tumor cell apoptosis compared with that in other groups (Figure 6C). Furthermore, the expressions of E-cadherin, FN-1 and Vimentin were determined, showing that compared with the sorafenib group, the level of E-cadherin was upregulated, and the expression levels of FN-1 and Vimentin were downregulated in the sorafenib plus LIMT over-expression group (Figure 6D). These data indicated that up-regulation of LIMT could inhibit tumor growth *in vivo*.

## Discussion

Sorafenib resistance and distant metastasis are closely correlated

with the poor prognosis in HCC patients. So, it is very important to reveal the underlying molecular mechanisms and explore novel therapeutic targets. Accumulated evidence indicates that lncRNAs regulate gene expression related to various processes, such as cell apoptosis, proliferation, and migration, suggesting that they may play a carcinogenic or tumor-suppressive role in cancers [20]. Furthermore, lncRNAs are involved in sorafenib resistance in various cancers [21]. LIMT, located on chromosome 12, was determined to be downregulated in cancers [9,22]. It had been reported that over-expression of LIMT could inhibit the proliferation, migration, and invasion of NSCLC cells by regulating the miR-152-3p/PTEN axis [23]. Furthermore, it has inhibitory effects on cell migration and metastasis in a variety of cancer cells, such as gastric cancer, colorectal cancer, breast cancer, and cervical cancer [9,22,24,25]. However, the role of LIMT in drug resistance has not been explored so far in HCC. Here, for the first time we explored the

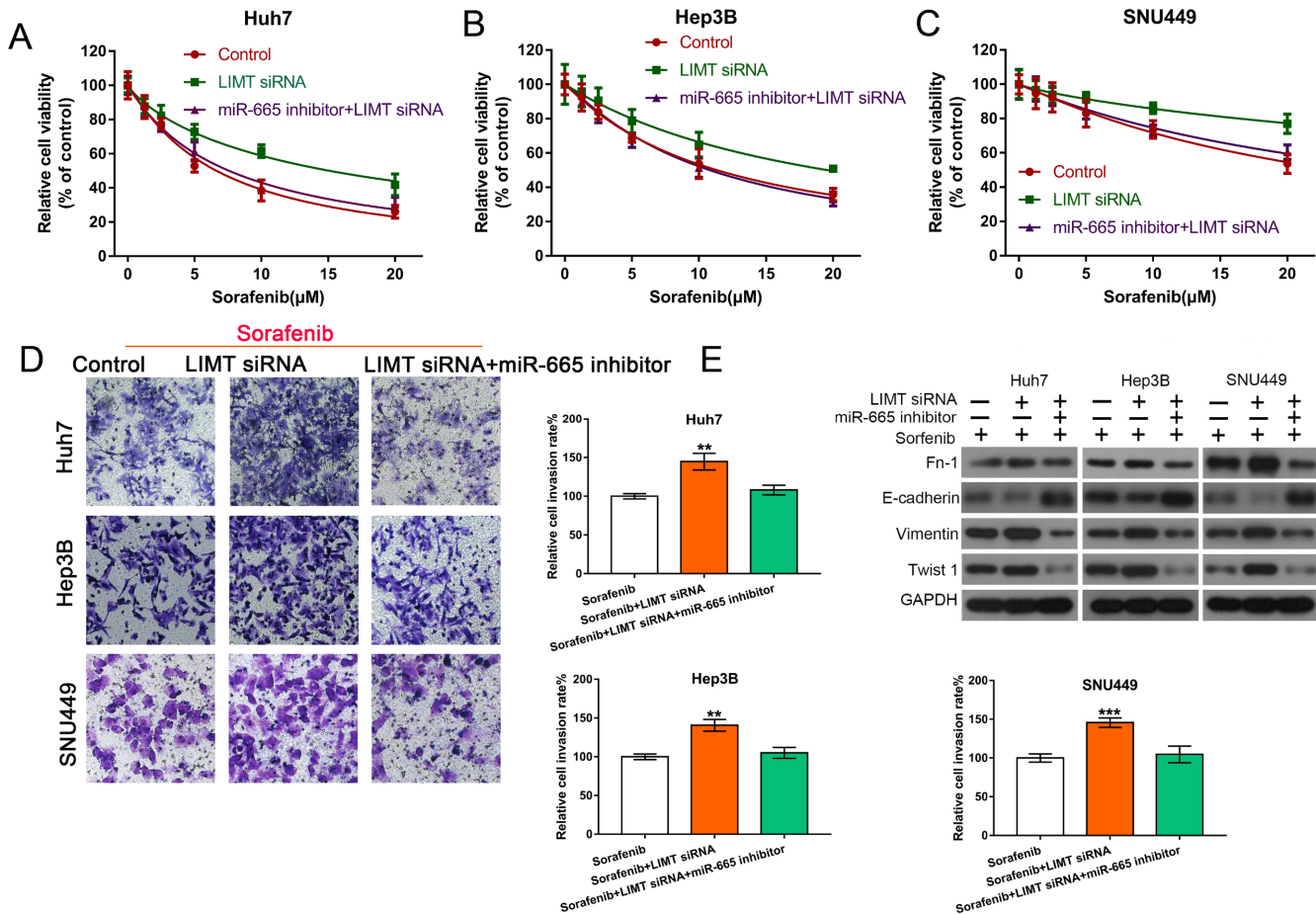


**Figure 4.** MiR-665 regulates sorafenib resistance in HCC cells (A,B) CCK-8 assay and EdU analysis were used to determine cell viability and proliferation of HCC cells exposed to sorafenib following miR-665 mimics or inhibitors treatment.  $**P < 0.01$  and  $***P < 0.001$ . (C) The invasion ability of HCC cells transfected with miR-665 mimics or inhibitors combined with sorafenib was confirmed by Transwell invasion analysis.  $*P < 0.05$ ,  $**P < 0.01$ , and  $***P < 0.001$ . (D) Western blot analysis of FN-1, E-cadherin, Vimentin and Twist 1 levels in HCC cells.

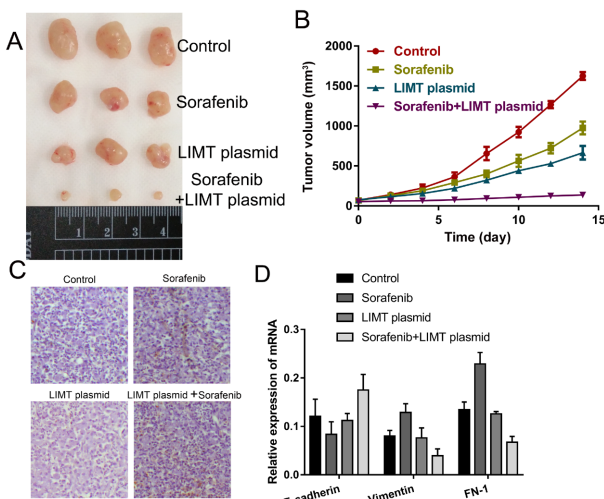
effect of LIMT on chemotherapy resistance in HCC. Our results verified that the level of LIMT was downregulated in HCC tumor tissue and HCC cells, implying that LIMT might play a role in suppressing cancers. As expected, knockdown of *LIMT* significantly increased sorafenib resistance and promoted cell invasion in HCC cells.

Evidence shows that some lncRNAs bind competitively to targeted miRNAs, thus acting as sponges of miRNA molecules. Silencing *SNHG16* inhibitor increased chemotherapy resistance by upregulating miR-140-5p in HCC [6]. Knockdown of *HOTAIR* enhanced sorafenib resistance by upregulating miR-217 in HCC [26]. Our study verified that downregulation of *LIMT* promoted sorafenib resistance by promoting EMT. The regulatory relationship between lncRNAs and miRNAs is related to many cell biological processes [27,28]. Thus, we further investigated the relationship between

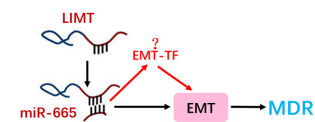
LIMT and miRNAs. We found that LIMT interacts directly with miR-665 to reduce its apparent level. Furthermore, several miRNAs have been proved to participate in sorafenib resistance in HCC. Upregulation of miR-223 could promote sorafenib resistance through regulation of FBW7 (encoding F-box and WD repeat domain containing 7) expression in HCC [29]. MiR-221 participates in sorafenib resistance [30]. Increasing evidence suggests that miR-665 is a tumor suppressor or cancer-promoting miRNA, which is expressed in tumor cells. For instance, miR-665 was reported to be a tumor-promoting factor in HCC. High miR-665 expression promotes cell metastasis, growth, and EMT of HCC and NSCLC cells [31–33]. Consistent with these previous reports, in this study we found that miR-665 mimics could enhance sorafenib resistance and invasion capability of HCC cells. Moreover, *LIMT* silencing abolished the effect of the miR-665 inhibitor on sorafenib resistance in HCC cells.



**Figure 5.** LIMT could regulate sorafenib resistance through regulating miR-665 and EMT (A–C) HCC cells were treated with the LIMT siRNA alone or combined with miR-665 inhibitor, following treatment with sorafenib, and cell viability was examined by CCK-8 assay. (D) The rate of invasive HCC cells treated with LIMT siRNA alone or combined with miR-665 inhibitor following treatment with sorafenib were determined by Transwell invasion analysis. \*\* $P < 0.01$  and \*\*\* $P < 0.001$ . (E) The levels of FN-1, E-cadherin, Vimentin and Twist 1 proteins were determined by western blot analysis.



**Figure 6.** LIMT over-expression could suppress tumor growth *in vivo* (A,B) Growth curves of xenograft tumors and the tumor size treated with control, sorafenib, LIMT plasmid, or sorafenib plus LIMT plasmid ( $n = 3$  per group). (C) TUNEL was used to analyze cell apoptosis in the treatment groups. (D) RT-qPCR was used to analyze the mRNA expression levels of E-cadherin, Vimentin and FN-1.



**Figure 7.** The mechanism of the LIMT in regulating resistance to sorafenib of HCC cells Schematic diagram of the regulatory mechanism of the LIMT/miR-665/EMT axis in regulating resistance to sorafenib of HCC cells.

Thus, *LIMIT* could regulate HCC cell sorafenib resistance. Recently, EMT has been found to be closely associated with the progress of chemoresistance, and reversing the formation of EMT could reduce chemotherapy resistance in various cancers, indicating that EMT is an effective therapeutic target for cancer metastasis and chemotherapy resistance [34,35]. It has been reported that TGF-β1/ROCK signaling pathway is involved in vasculogenic mimicry formation by inducing EMT [36]. For instance, a previous study demonstrated that H19 could promote paclitaxel resistance by adjusting miR-340 expression and EMT in breast cancer cells [37]. Upregulation of miR-206 could inhibit cell proliferation, migration and promote apoptosis in HCC cells by modulating cMET expression [38]. Over-expression of miR-4458 could also inhibit the migration, invasion, and EMT of HCC cells by suppressing the TGF-β



signaling [39]. Our study also revealed that inhibition of LIMT could affect sorafenib resistance and promote cell invasion by regulating EMT. EMT-induced drug resistance in cancer cells involves many typical signaling pathways, such as Wnt/ $\beta$ -catenin, nuclear factor kappa B (NF- $\kappa$ B), and phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/protein kinase B (AKT) [40–42]. Therefore, the relationship between the LIMT and EMT-related signaling pathways still needs further investigation in the future.

In summary, the present study revealed that regulation of LIMT is a mechanism involved in the resistance to sorafenib directly through regulating miR-665 and EMT in HCC cells (Figure 7). Our results demonstrated that down-regulation of LIMT could enhance sorafenib resistance and promote EMT by regulation of miR-665, suggesting that LIMT might serve as a molecular target for sorafenib resistance detection treatment and prognosis in HCC by regulating the miR-665/EMT axis.

### Supplementary Data

Supplementary data is available at *Acta Biochimica et Biophysica Sinica* online.

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### Conflict of Interest

The authors declare that they have no conflict of interest.

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