Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;51:1179-1192
DOI: 10.1159/000495495
Published online: 24 November 2018
Accepted: 16 November 2018

Original Paper

miR-650 Promotes the Metastasis and Epithelial–Mesenchymal Transition of Hepatocellular Carcinoma by Directly Inhibiting LATS2 Expression

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Key Words
EMT • Hepatocellular carcinoma • LATS2 • miR-650 • YAP

Abstract
Background/Aims: Previous studies have confirmed that microRNAs are involved in the metastasis and epithelial–mesenchymal transition (EMT) of malignancies. In this study, we examined whether miR-650 promotes the migration, invasion, and EMT of hepatocellular carcinoma (HCC) cells by targeting the large tumor suppressor kinase 2 gene (*LATS2*). ***Methods:*** qRT-PCR was used to detect expression of miR-650 in HCC tissues and paired normal tissues. MTT and Transwell assay were used to observe the effect of miR-650 on proliferation, migration and invasion of HCC cells. Western blot assay and Immunohistochemistry were performed to demonstrate association between miR-650 expression level and epithelial-mesenchymal transition (EMT) related protein. Mechanistically, Reporter luciferase assay was performed to reveal whether large tumor suppressor kinase 2 (LATS2) was a direct target of miR-650 in HCC cells. ***Results:*** We observed that miR-650 levels were largely up-regulated in HCC tissues, and that the increased expression was closely associated with the adverse clinical features of HCC patients. Additionally, the expression of *LATS2*, which was identified as a direct target of miR-650, can counteract the effects of miR-650 in HCC. Furthermore, we demonstrated that high miR-650 expression levels and low *LATS2* expression levels in tumors may indicate a poor prognosis for HCC patients. ***Conclusion:*** In conclusion, the miR-650/*LATS2* pathway may serve as a novel prognostic biomarker and an attractive therapeutic target for HCC patients.

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Introduction

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related deaths worldwide [1]. The prognosis for HCC patients is currently extremely poor because of the highly invasive nature of the disease [2]. Additionally, the mechanisms underlying HCC metastasis have not been accurately characterized. The epithelial–mesenchymal transition (EMT), which involves the conversion of a cancer cell from its epithelial form to its motile mesenchymal form, is recognized as an initial and essential event in the metastatic progression of various carcinomas [3, 4]. MicroRNAs (miRNAs) are a class of small non-coding RNAs that serve as post-transcriptional regulators mainly by targeting and degrading specific mRNAs [5]. miRNAs function as crucial modulators that regulate many cancer-related biological processes, including invasion, metastasis, and EMT [6]. Many miRNAs are involved in the metastasis and EMT of HCC. Recent research has focused on the effect of miR-650 on the progression of human cancers [7, 8]. The miR-650 genomic sequence is associated with the Ig λ variable region gene [9]. Interestingly, miR-650 may have different roles in diverse cancers. For example, in chronic lymphocytic leukemia, miR-650 is positively associated with a favorable prognosis [10], while miR-650 can promote the progression of gastric cancer by stimulating metastasis [11]. Additionally, Zheng et al [12]. reported that increased expression of miR-650 is associated with the progression of HCC. However, no causal relationship has been established between miR-650 and HCC metastasis and EMT. Therefore, further research into miR-650 functions related to HCC progression is necessary.

In this study, we analyzed HCC tissue samples and revealed that miR-650 functions as a prognostic indicator. The effects of miR-650 on the growth, migration, invasion, and EMT of HCC cells and the possible underlying mechanisms were also investigated.

Materials and Methods

Patients and tissue samples

The 130 tissue samples analyzed in this study were collected from HCC patients who underwent a surgical resection at The Second Affiliated Hospital of Xi'an Jiaotong University between March 2010 and March 2011. The clinical–pathological characteristics of all patients are listed in Table 1. None of the patients underwent prior chemotherapy or radiotherapy. Fresh HCC tissue samples and paired distant non-cancerous tissue samples (≥2 cm from the margin) were immediately frozen in liquid nitrogen during the surgical resection and stored at −80°C (for a western blot or qRT-PCR assay) or in paraffin (for an immunohistochemistry [IHC] assay). This study was approved by the Ethics Committee of Clinical Research of Xi'an Jiaotong University.

Table 1. Association between miR-650 expression and clinical–pathological features in HCC (n = 130)

Variable	Total no. of patients n=130	miR-650		p
		Low expression	High expression	
Age (years)				0.348
<50	65(50.0%)	24	41	
≥50	65(50.0%)	18	47	
Gender				0.223
Female	26(20.0%)	11	15	
Male	104(80.0%)	31	73	
HBsAg				0.832
Positive	107(82.3%)	35	72	
Negative	23(17.7%)	7	16	
AFP (ng / mL)				0.272
<400	53(40.8%)	20	33	
≥400	77(59.2%)	22	55	
Cirrhosis				0.554
Yes	76(58.5%)	23	53	
No	54(41.5%)	19	35	
Tumor size (cm)				0.003**
<5	75(57.7%)	32	43	
≥5	55(42.3%)	10	45	
Tumor multiplicity				0.647
Single	111(85.4%)	35	76	
Multiple	19(14.6%)	7	12	
Differentiation				0.885
Well–moderate	60(46.2%)	19	41	
Poor–undifferentiation	70(53.8%)	23	47	
Microscopic vascular invasion				<0.001**
Yes	49(37.7%)	26	23	
No	91(62.3%)	16	65	
Stage				0.043*
I-II	83(63.8%)	32	51	
III-IV	47(36.2%)	45 10	37	

qRT-PCR assay

The primers used in the qRT-PCR assay were designed and synthesized by Takara (Dalian, China), and the sequences are provided in Table 2. The qRT-PCR assay was conducted in triplicate. The *U6* RNA level was used as the internal control for miR-650, while glyceraldehyde 3-phosphate dehydrogenase

(*GAPDH*) was used as the internal control for large tumor suppressor kinase 2 (*LATS2*) and the other genes. The gene expression levels relative to the *GAPDH* expression level were assessed using the $2^{-\Delta\Delta Ct}$ method.

Table 2. Sequences of the qRT-PCR primers

Variable	Forward	Reverse
miR-650	5'-AGAGGAGGCAGCGCTCT-3'	5'-CAGTGCGTGTCGTGGAGT-3'
LATS2	5'-ACCCCAAAGTTCGGACCTTAT-3'	5'- CATTTGCCGGTTCACTTCTGC-3'
Cyr61	5′-CCCTGAACTTGTGGATGTCATTG-3′	5′-GTCATGATGATCCAGTCCTGCAAA-3′
AREG	5′-TGCTGGATTGGACCTCAATG-3′	5′-TCCCGAGGACGGTTCACTAC-3′
CTGF	5′-GAAAAGAUUCCCACCCAU-3′	5′-AUUGGGUGGGAAUCUUUUC-3′
CXCL5	5′-GTTCCATCTCGCCATTCATGC-3′	5′-GCGGCTATGACTGAGGAAGG-3′
GAPDH	5'-AATGGACAACTGGTCGTGGAC-3'	5'-CCCTCCAGGGGATCT GTTTG-3'

IHC assay

An IHC assay was performed as previously described [13]. The antibodies used in this study included the anti-LATS2 antibody (bs-4081R, Beijing Bioss Biotechnology, Beijing, China), anti-E-cadherin antibody (cat. bs-1016R, Beijing Bioss Biotechnology), and anti-vimentin antibody (cat. bs-8533R, Beijing Bioss Biotechnology).

Cell culture and transfection

The HCC and LO2 cell lines were purchased from the American Type Culture Collection (Manassas, VA) and were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin, and 100 units/ml penicillin (Sigma-Aldrich, St. Louis, MO) in an incubator set at 37°C with 5% CO_2. miR-650 mimic was acquired from Genecopoeia (Guangzhou, China), while the LATS2 vector was obtained from Addgene (Cambridge, MA). Additionally, an anti-miR-650 inhibitor and LATS2 small interfering RNA (siRNA) were purchased from Origene (Beijing, China). Cells were transfected with these vectors and siRNA using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's protocol.

Western blot assay

The cells of each line were lysed in RIPA Buffer (cat. 9800, Cell Signaling Technology, Danvers, MA) to extract proteins, after which the protein concentrations were determined in bicinchoninic acid assays (cat. 7780, Cell Signaling Technology). For each sample, the total protein content was quantified with the Bradford method. Total proteins (25 μg) were resolved by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and subsequently transferred to polyvinylidene fluoride membranes. The membranes were blocked in 5% non-fat milk for 1 h and then probed with the following primary antibodies: anti-LATS2 antibody (cat. bs-4081R; Beijing Bioss Biotechnology, Beijing, China), anti-yes-associated protein (YAP) antibody (cat. 14074, Cell Signaling Technology), anti-phospho-YAP (Ser127) antibody (cat. 13008, Cell Signaling Technology), anti-E-cadherin antibody (cat. 3195, Cell Signaling Technology), anti-vimentin antibody (cat. 5741, Cell Signaling Technology), and anti-GAPDH (cat. ab6922; Abcam, MA, USA). The membranes were incubated at 4°C overnight, washed, and then incubated with anti-rabbit secondary antibodies for LATS2, YAP, phosphorylated YAP (Ser127), E-cadherin, vimentin, and an anti-mouse secondary antibody for GAPDH at 37°C for 2 h. Finally, the membranes were analyzed with a bio-imaging system (DNR Bio-Imaging Systems, Jerusalem, Israel).

Cell migration and invasion assays

The migration and invasive ability of HCC cells were measured in 24-well Transwell plates (Corning Costar, Tewksbury, MA). For the cell migration assays, 2×10^4 HCC cells in 200 μl FBS-free medium were uniformly implanted in the upper chamber, while 600 μl normal culture medium (with 10% FBS) was added to the bottom chamber. After a 48-h incubation, the cells remaining on the membrane in the upper chamber were carefully removed with a cotton swab, while the cells that migrated through the membrane to the surface of the bottom chamber were gently cleaned and fixed with methanol. The migratory cells were stained with crystal violet solution and air-dried at 37°C. The migratory cells in 10 random microscopic fields were counted. For the invasion assay, we performed the same procedures as described above except we also coated the membrane in the upper chamber with 200 mg/ml Matrigel (BD Biosciences, San Jose, CA).

MTT assays

MHCC97-H or HepG2 cells (500 cells/well in 100 µl medium) were added to a 96-well plate and incubated at 37°C for 24, 48, 72, 96, 120, 144, and 168 h. The medium was replaced with fresh medium every 48 h for samples incubated for 72, 96, 120, 144, and 168 h. At each analyzed time point, 10 µl MTT solution (5 mg/ml in PBS) was added to each well. After a 4-h incubation at 37°C, the medium was removed, and 200 µl dimethyl sulfoxide was added. Cell viability was assessed using a plate reader (492 nm wavelength) (Bio-Rad Laboratories, Hercules, CA). Analyses were conducted in triplicate.

Luciferase reporter assay

The indicated cells were added to a 24-well plate and transfected with miR-650, the control, or 3′-untranslated region (UTR)-luciferase plasmids. Cells were collected at 48 h after the transfection, and luciferase activity was measured using a Dual-Luciferase Assay System (Promega, Madison, WI).

Statistical analysis

Statistical analyses were performed using SPSS 20.0 (SPSS Inc., Chicago, IL). All experiments were repeated three times. The data are presented as the mean ± standard deviation. The chi-square test and Fisher's exact test (two-sided) were conducted to assess the association between clinical–pathological characteristics and gene expression. Spearman correlation analysis was used to examine the correlation between the expression levels of two genes. The Kaplan–Meier method (log-rank test) was completed to plot the survival curves of different sub-groups. A p-value less than 0.05 in a two-sided test was applied as the threshold for significance.

Results

Overexpression of miR-650 is associated with HCC progression

qRT-PCR analysis revealed that miR-650 expression levels were considerably higher in HCC tissue samples than in the paired normal tissues (5.38 ± 0.33 vs. 2.98 ± 0.12, $p < 0.01$, Fig. 1A). A subsequent analysis suggested that miR-650 expression was significantly higher in HCC tissues from patients with metastasis than in patients without metastasis (6.12 ± 0.21 vs. 4.18 ± 0.17, $p < 0.01$, Fig. 1B). We also evaluated the clinical significance of miR-650 in HCC patients. The data indicated that high miR-650 expression levels were associated with microscopic vascular invasion ($p < 0.001$), a relatively large tumor ($p = 0.003$), and an advanced TNM stage in HCC patients ($p = 0.043$) (Table 1).

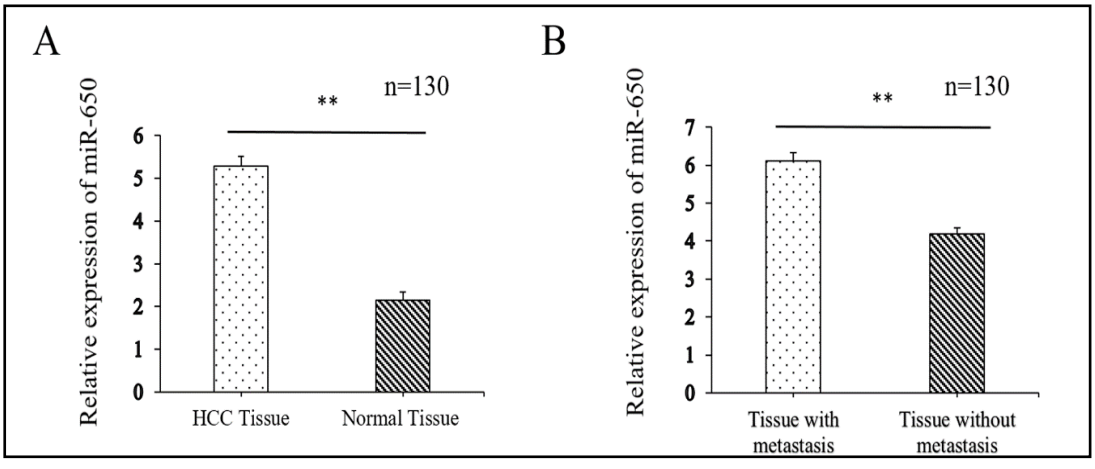

Fig. 1. Expression of miR-650 in HCC tissues. A. Relative miR-650 expression levels were compared between HCC tissues and paired distant non-cancerous tissues (n = 130, **p<0.01). B. Comparison of relative miR-650 expression levels in tumor samples from patients with and without metastasis (n = 130, **p<0.01). Target gene expression levels were quantified relative to GAPDH expression levels.

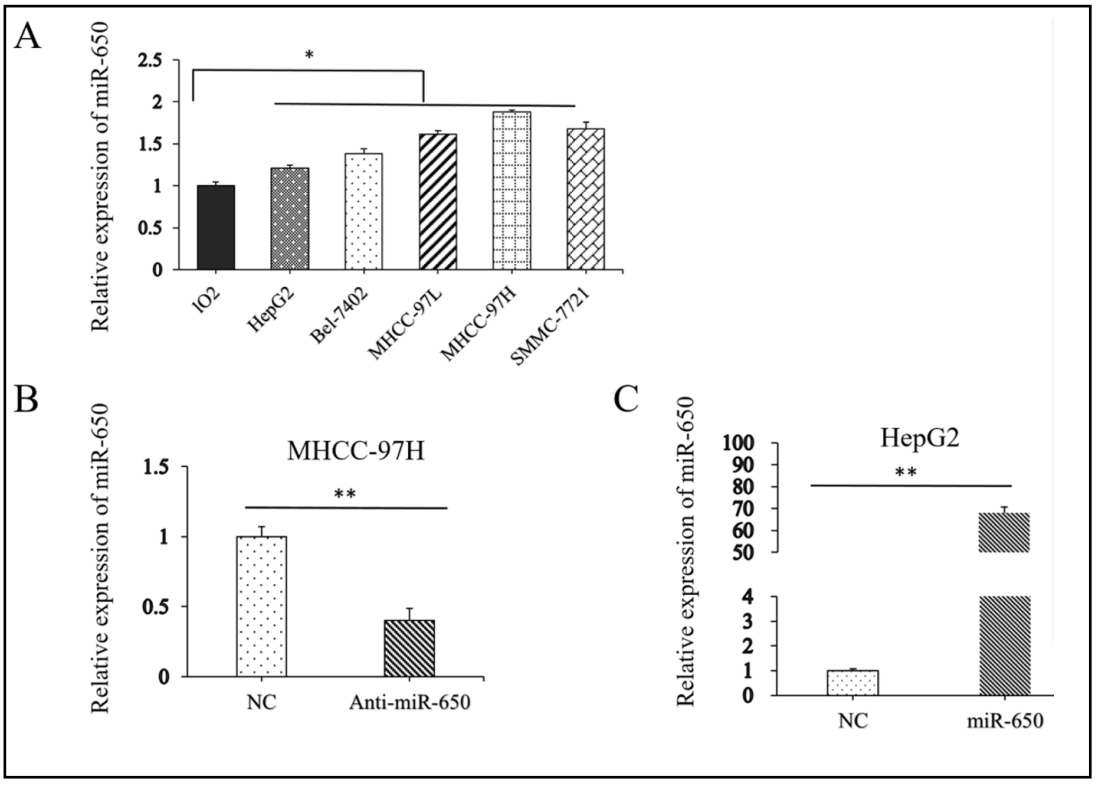

Fig. 2. Expression of miR-650 in HCC cells. A. miR-650 expression levels in various human HCC cell lines and human hepatocyte LO2 cells (*p<0.05). B. miR-650 expression level significantly decreased after MHCC-97H cells were transfected with the miR-650 inhibitor (**p<0.01). C. miR-650 expression level significantly increased after HepG2 cells were transfected with the miR-650 mimic (**p<0.01).

miR-650 promotes HCC cell migration and invasion

To evaluate the function of miR-650 in HCC cells, we compared the miR-650 expression levels in HCC cell lines and the human liver cell line LO2. Consistent with the data for the HCC samples, the qRT-PCR analysis confirmed that miR-650 expression levels were markedly higher in HCC cells than in LO2 cells ($p < 0.05$, Fig. 2A). Among the HCC cell lines, the highest and lowest miR-650 expression levels were observed in MHCC-97H and HepG2 cells, respectively. We then transfected MHCC97-H and HepG2 cells with the miR-650 inhibitor and the miR-650 mimic, respectively. The miR-650 inhibitor decreased the miR-650 expression level in MHCC97-H cells ($p < 0.01$, Fig. 2B), while the miR-650 mimic considerably increased the miR-650 expression level in HepG2 cells ($p < 0.05$, Fig. 2C). Analyses of cell migration and invasion suggested that decreased miR-650 levels markedly inhibited the migration and invasion capacity of MHCC-97H cells ($p < 0.01$, Fig. 3A). In contrast, the increased miR-650 expression in HepG2 cells significantly enhanced the migration and invasion ability of cells ($p < 0.01$, Fig. 3B). Our results demonstrated that miR-650 promotes HCC cell migration and invasion.

miR-650 promotes HCC EMT

We further investigated whether miR-650 helps mediate HCC EMT. The western blot data revealed that decreased miR-650 levels resulted in increased E-cadherin expression (epithelial marker) and decreased vimentin and Snail expression (mesenchymal markers) in MHCC-97H cells. The opposite trends were observed when miR-650 levels increased ($p < 0.01$, Fig. 4A). To evaluate whether miR-650 promotes HCC EMT, we conducted an IHC assay to compare the E-cadherin and vimentin levels in HCC tissues ($p < 0.01$, Fig. 4B). Spearman

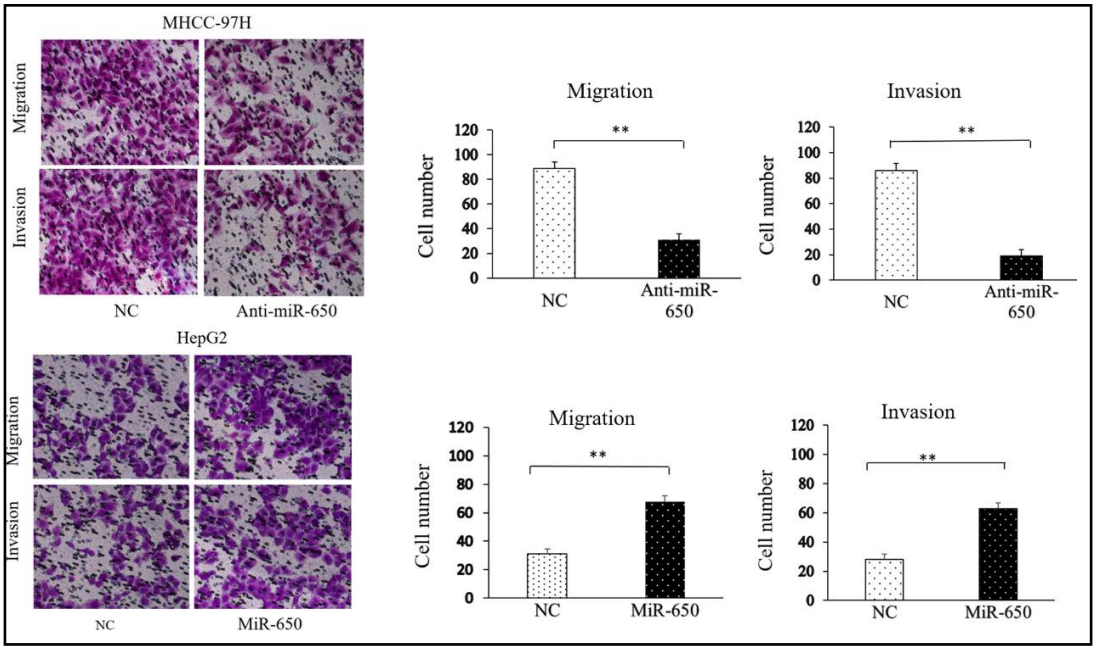

Fig. 3. Analysis of cell migration and invasion using 24-well Transwell plates indicated miR-650 expression affected HCC cell invasion and migration. The invaded cells were quantified by counting the cells in 10 random fields (magnification 200×). Data are presented as the mean ± standard deviation of three independent experiments.

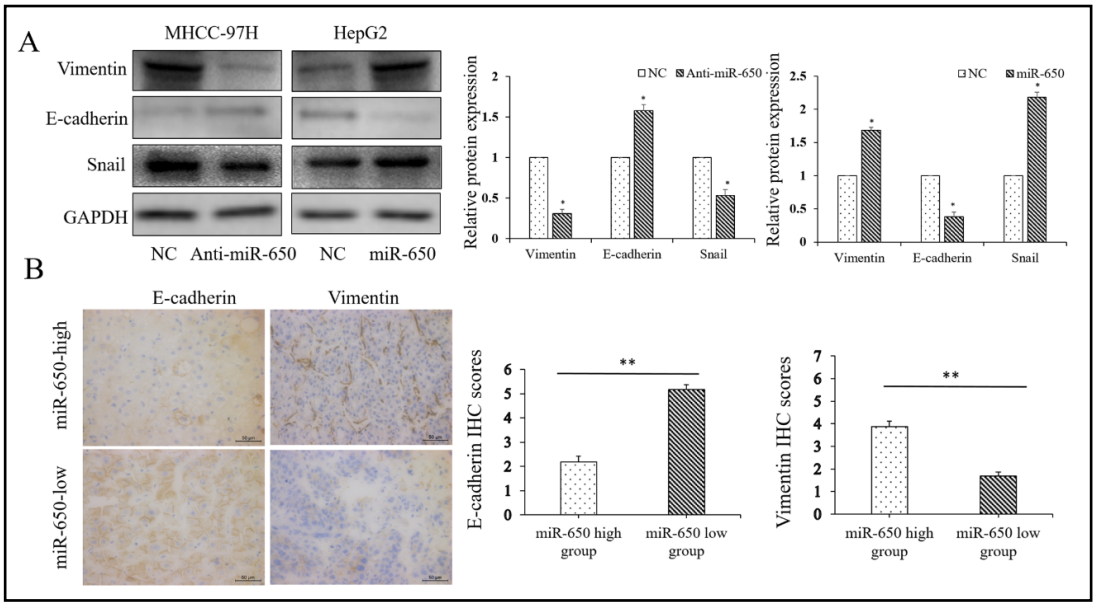

Fig. 4. miR-650 promotes HCC EMT. A. Western blot analysis of E-cadherin, vimentin, and Snail in MHCC-97H cells in which miR-650 expression was down-regulated and HepG2 cells in which miR-650 expression was up-regulated (*p<0.05). B. IHC analysis of E-cadherin and vimentin levels in tissues with high and low miR-650 expression levels (n = 130, *p<0.01) (400×).

correlation analysis confirmed that the miR-650 expression level was negatively associated with the E-cadherin expression level ($p < 0.001$, $r = -0.468$), but positively associated with the vimentin expression level ($p < 0.001$, $r = 0.275$; Table 3). These results implied that miR-650 promotes HCC EMT.

LATS2 may be directly targeted by miR-650 in HCC cells

To explore the potential mechanism underlying the miR-650 function in HCC cells, we searched the TargetScan public database for genes targeted by miR-650. We detected a sequence complementary to miR-650 in the 3′-UTR of *LATS2* mRNA (Fig. 5A). Because LATS2 is a critical regulator of cancer metastasis via its effects on the Hippo signaling pathway, which is important for regulating EMT [14], we examined whether miR-650 promotes HCC EMT through LATS2. Specifically, we performed an IHC assay to assess the LATS2 abundance in HCC tissues and paired distant non-cancerous tissues. We observed that LATS2 levels were markedly lower in HCC tissues than in non-cancerous tissues (2.14 ± 0.12 vs. 4.83 ± 0.24, $p < 0.01$, Fig. 5B). Moreover, *LATS2* levels were considerably lower in samples from patients with metastasis than in samples from

Table 3. Association between miR-650 and EMT-related protein expression in HCC tissue samples. *p < 0.05; **p < 0.01; n = 130

Variable	miR-650		r	p
	High expression	Low expression		
LATS2				
Tumor				
High expression	30	28	-0.306	<0.001**
Low expression	58	14		
Normal			-0.122	0.167
High expression	29	34		
Low expression	39	28		

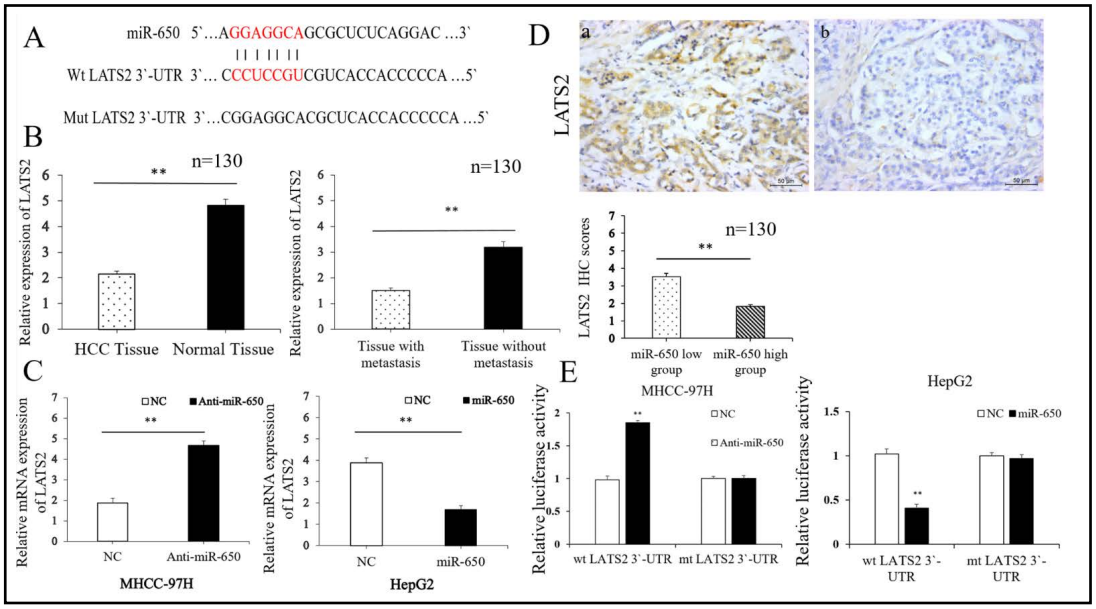

Fig. 5. LATS2 is a direct downstream target of miR-650 in HCC. A. miR-650 and its putative binding sequence in the 3′-UTR of LATS2. B. Comparison of relative LATS2 expression levels in HCC tissues and distant non-cancerous tissues (n = 130, **p<0.01). Comparison of relative LATS2 expression levels in tumor samples from patients with and without metastasis (n = 130, **p<0.01). C. LATS2 expression level significantly increased after MHCC-97H cells were transfected with the miR-650 inhibitor (**p<0.01). The LATS2 expression level significantly decreased after HepG2 cells were transfected with the miR-650 mimic (**p<0.01). D. IHC analysis of LATS2 in tissues with low (a) and high (b) miR-650 levels (n = 130, *p<0.01). E. Increased or decreased miR-650 levels significantly affected luciferase activity in HCC cells carrying wild-type LATS2, but not in HCC cells carrying LATS2 with a mutated 3′-UTR (**p<0.01).

patients without metastasis (1.51 ± 0.09 vs. 3.19 ± 0.21, p < 0.01, Fig. 5B). Furthermore, we completed a qRT-PCR assay to examine the *LATS2* expression level in miR-650 knockdown and overexpression HCC cells. Decreased miR-650 levels in MHCC-97H cells up-regulated *LATS2* expression. However, increased miR-650 levels in HepG2 cells markedly down-regulated *LATS2* expression (p < 0.01, Fig. 5C). Additionally, the IHC assay results (Fig. 5D) also indicated that LATS2 levels were markedly lower in HCC tissues in which miR-650 was highly expressed than in HCC tissues in which miR-650 was expressed at relatively low levels (p < 0.01). Statistical analysis revealed an inverse correlation between a high miR-650 expression level and a low *LATS2* expression level in HCC tissue samples (r = −0.306). In contrast, a significant inverse association was not observed between *LATS2* and miR-650 expression levels in non-cancerous tissue samples (p > 0.05; Table 4). To confirm that miR-650 binds to *LATS2* to exert its effects in HCC cells, we completed luciferase reporter assays. Decreased miR-650 levels markedly promoted the luciferase activity associated with *LATS2* with a wild-type 3′-UTR in MHCC-97H cells (Fig. 5E). Meanwhile, increased miR-650 levels in HepG2 cells inhibited the luciferase activity associated with *LATS2* with a wild-type 3′-UTR. Additionally, altering miR-650 expression did not significantly affect the luciferase activity associated with *LATS2* with a mutated 3′-UTR. These findings suggested that *LATS2* may be directly targeted by miR-650 in HCC cells.

Table 4. Association between miR-650 and LATS2 expression in HCC tissue samples. *p < 0.05; **p < 0.01; n = 130

	miR-650			
Variable	High expression	Low expression	r	p
E-cadherin			-0.468	p<0.001**
High expression	20	31		
Low expression	68	11		
Vimentin				
High expression	61	16	0.297	0.001*
Low expression	27	26		

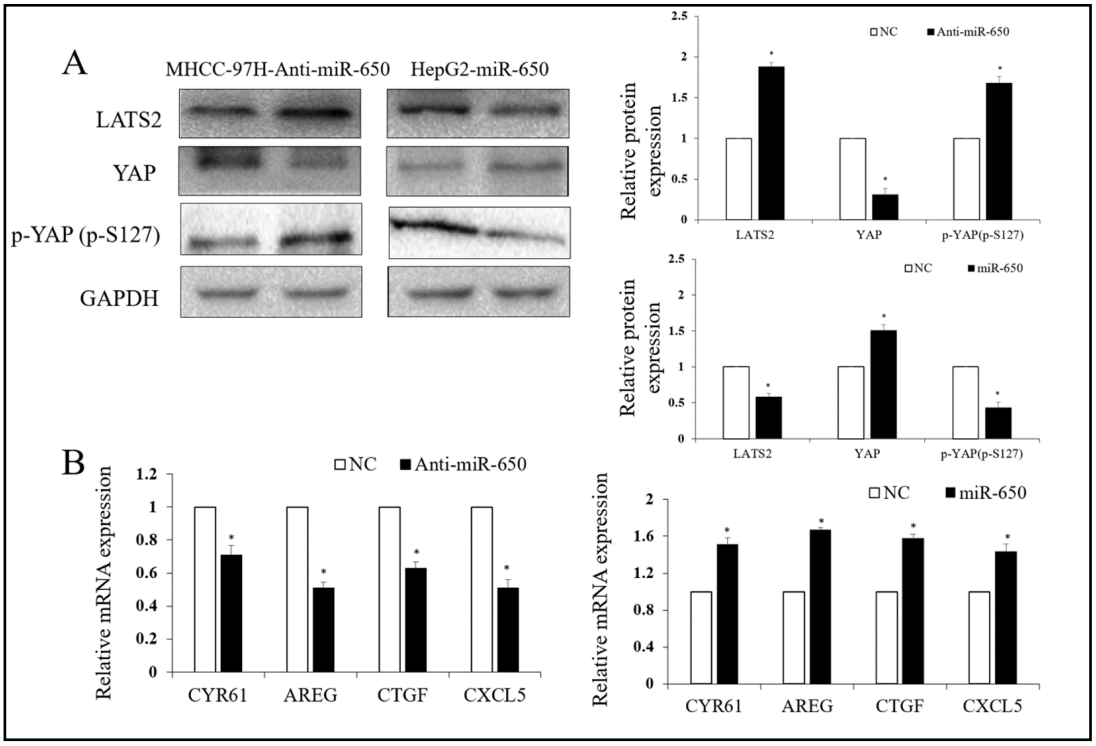

Fig. 6. Changes in miR-650 expression regulated the LATS2/YAP signaling pathway. A. Down-regulation or overexpression of miR-650 regulated the abundance of LATS2, YAP, and phosphorylated YAP (p-YAP [p-S127]) (*p<0.05). B. qRT-PCR analysis revealed that down-regulation or overexpression of miR-650 affected the expression of downstream YAP target genes, including CYR61, AREG, CTGF, and CXCL5 (*p<0.05).

MiR-650 regulates YAP and its downstream target genes

A previous study revealed that the LATS2 kinase phosphorylates the Hippo downstream effector YAP to regulate HCC EMT [15]. Therefore, we explored the effect of miR-650 on YAP by measuring the LATS2, YAP, and phosphorylated YAP (Ser127) levels. Decreased miR-650 levels significantly increased LATS2 levels (Fig. 6A). In contrast, miR-650 overexpression significantly decreased LATS2 levels ($p < 0.05$, Fig. 6A). Additionally, the phosphorylated YAP (Ser127) levels were measured using phospho-specific antibodies that recognize the LATS2 phosphorylation site on YAP (Ser127) [16]. Decreased miR-650 levels considerably increased the phosphorylated YAP (Ser127) levels, while increased miR-650 levels had the opposite effect ($p < 0.05$, Fig. 6A). Furthermore, qRT-PCR analysis indicated that some downstream YAP target genes (*CYR61*, *AREG*, *CXCL5*, and *CTGF*) were also regulated by miR-650 (Fig. 6B). Thus, we demonstrated that miR-650 serves as an upstream LATS2/YAP signal that helps regulate YAP and its downstream target genes.

LATS2 counteracts the miR-650 function in HCC cells

To evaluate the effects of LATS2 on the ability of miR-650 to promote metastasis, growth, and HCC EMT, MHCC-97H cells in which miR-650 levels were down-regulated and HepG2 cells in which miR-650 levels were up-regulated were treated with LATS2 siRNA and the LATS2 plasmid, respectively. Decreased LATS2 levels lessened the inhibition of the EMT due to the miR-650 inhibitor in MHCC-97H cells ($p < 0.05$) (Fig. 7A). Meanwhile, increased LATS2 levels reversed the promotion of EMT induced by miR-650 overexpression in HepG2 cells ($p < 0.05$, Fig. 7A).

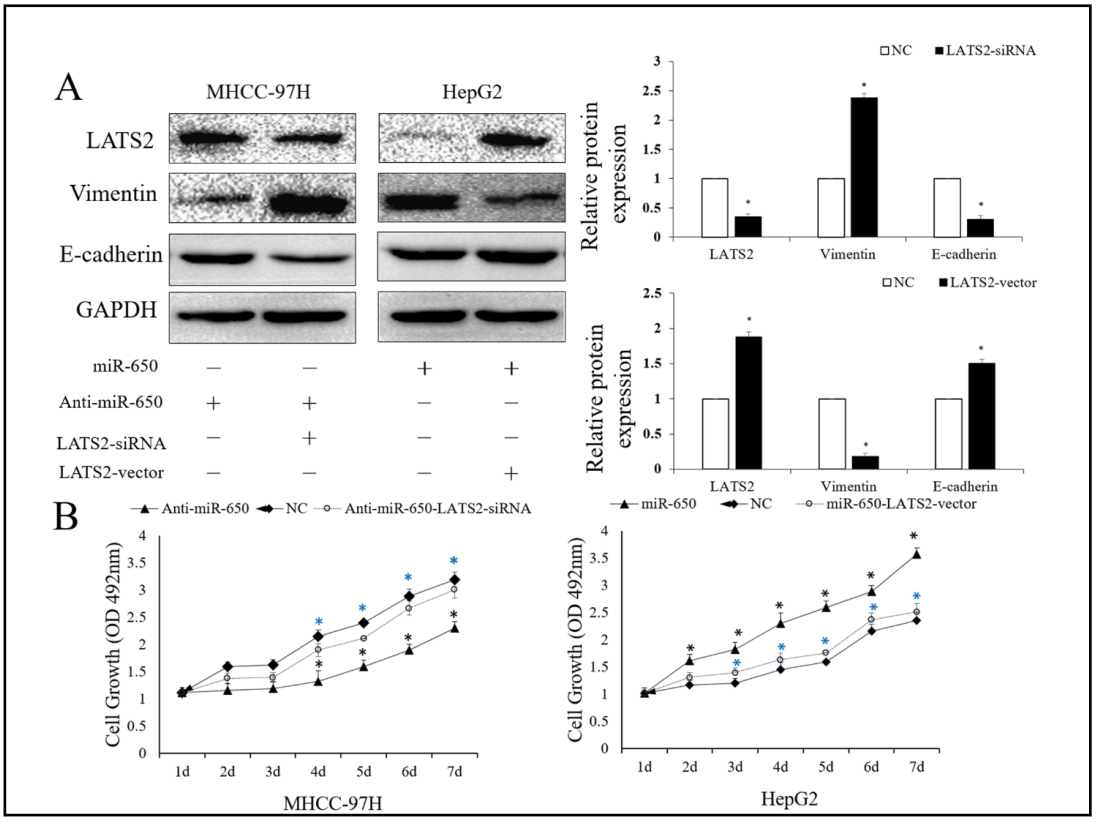

Fig. 7. LATS2 mediates the functions of miR-650 in HCC cells (*p<0.05). A. Western blot analysis indicated LATS2 mediates the effects of miR-650 on HCC EMT (*p<0.05). B. MTT assay confirmed that MHCC-97H cell proliferation decreased after miR-650 expression was down-regulated, while HepG2 cell proliferation increased after miR-650 expression was up-regulated (*p<0.05). LATS2 mediates the effects of miR-650 on HCC cell proliferation (*p<0.05).

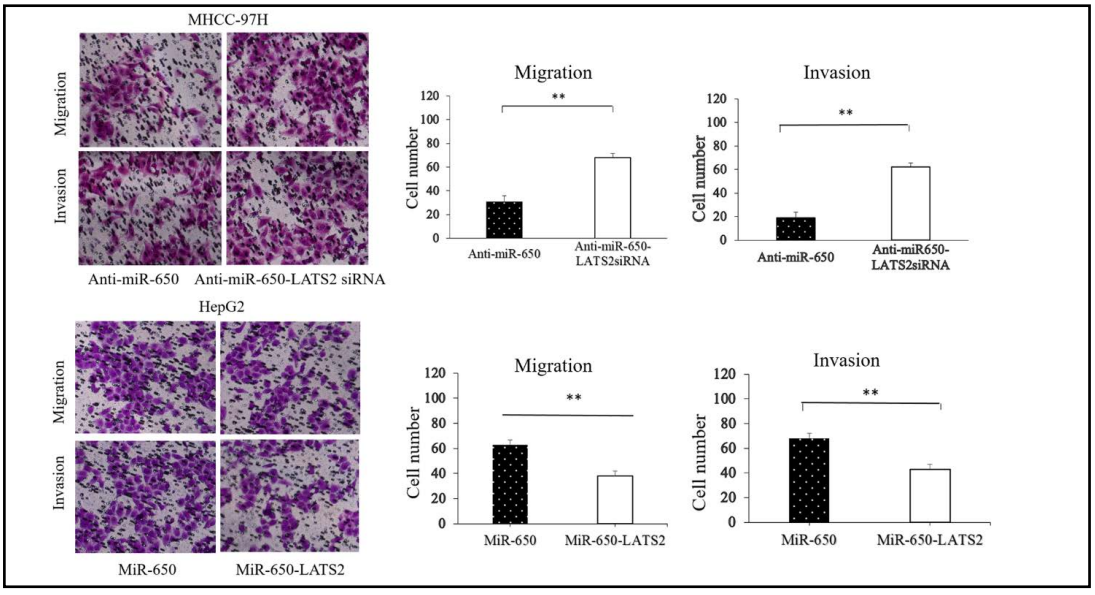

Fig. 8. Analysis of cell migration and invasion using 24-well Transwell plates revealed LATS2 mediates the effects of miR-650 on HCC cell migration and invasion (*p<0.01). The invaded cells were quantified by counting the cells in 10 random fields (magnification 200×). Data are presented as the mean ± standard deviation of three independent experiments.

Our MTT assay revealed that decreased miR-650 levels in MHCC-97H cells resulted in a diminished ability of cells to proliferate. In contrast, increased miR-650 levels in HepG2 cells significantly enhanced cell proliferation. The MTT assay also indicated that altering LATS2 expression could counteract the cell proliferation induced by miR-650 ($p < 0.01$, Fig. 7B).

Analyses of cell migration and invasion indicated that decreased LATS2 levels diminished the inhibited cell migration and invasion induced by decreased miR-650 levels in MHCC-97H cells, while increased LATS2 levels rescued the cell migration and invasion ability of HepG2 cells promoted by increased miR-650 levels ($p < 0.01$, Fig. 8). These results implied that LATS2 is an important determinant for the properties of miR-650 in HCC metastasis and EMT.

Prognostic significance of miR-650 and LATS2 expression for HCC patients

We evaluated the prognostic significance of miR-650 and *LATS2* expression in HCC patients. An analysis using the Kaplan–Meier method revealed that high miR-650 expression levels were closely correlated with shorter overall survival (OS) and disease-free survival (DFS) ($p < 0.01$, Fig. 9A and B). Additionally, *LATS2* expression was significantly associated with improved OS and DFS ($p < 0.01$, Fig. 9C and D). Further analyses confirmed that HCC patients with high miR-650 expression levels and low *LATS2* expression levels had the worst OS and DFS. In contrast, simultaneously low miR-650 expression levels and high *LATS2* expression levels resulted in the best OS and DFS for HCC patients ($p < 0.01$, Fig. 9E and F). Thus, the Kaplan–Meier analysis revealed a combination of miR-650 and *LATS2* expression levels may be useful for determining the prognosis for HCC patients.

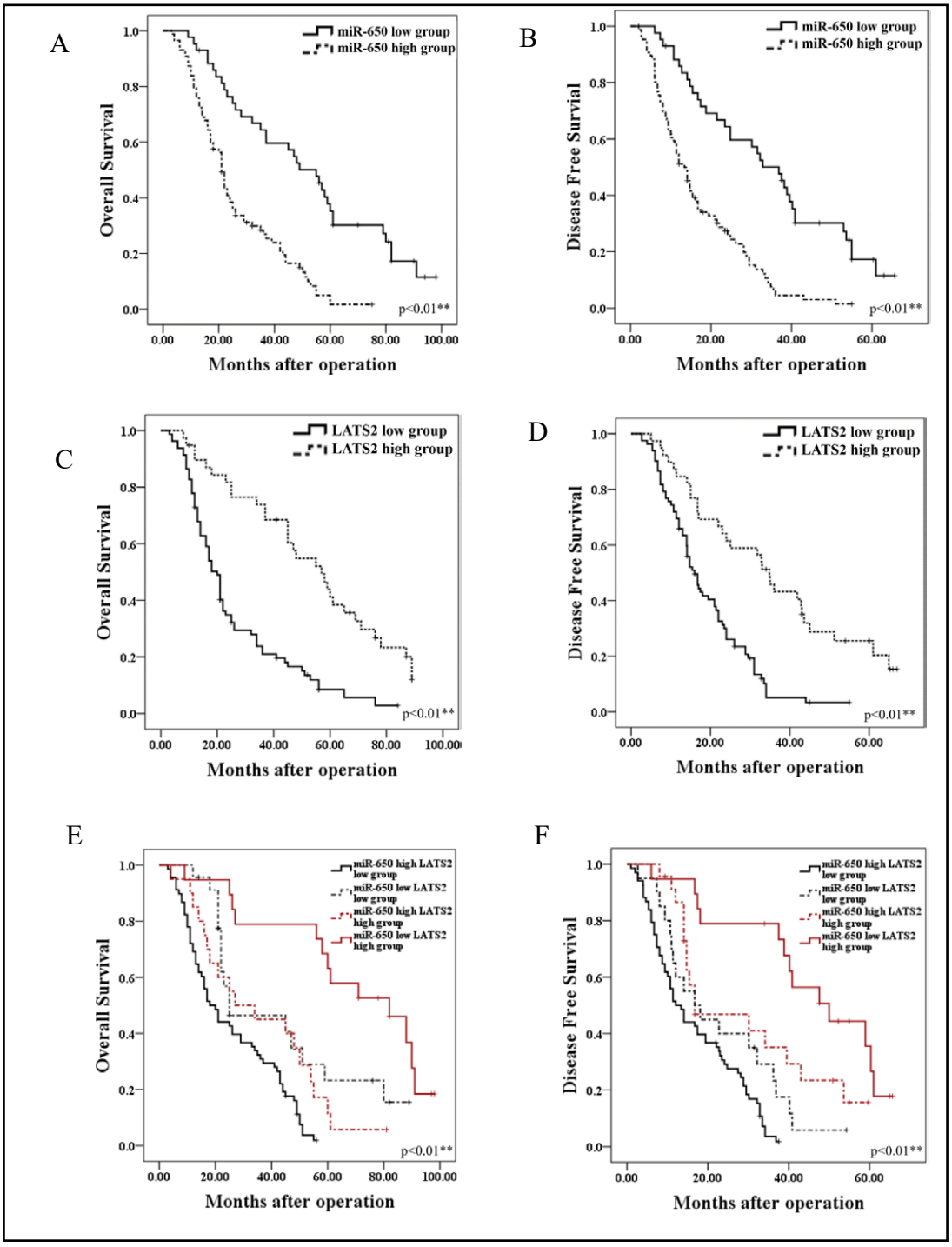

Fig. 9. Prognostic value of miR-650 and LATS2 expression for HCC patients as determined by a Kaplan–Meier analysis. A. Comparison of OS between HCC patients with high and low miR-650 expression levels. B. Comparison of DFS between HCC patients with high and low miR-650 expression levels. C. Comparison of OS between HCC patients with high and low LATS2 expression levels. D. Comparison of DFS between HCC patients with high and low LATS2 expression levels. E. Comparison of OS among four subgroups of HCC patients (high miR-650/low LATS2 expression; low miR-650/low LATS2 expression; high miR-650/high LATS2 expression; low miR-650/high LATS2 expression). F. Comparison of DFS among four subgroups of HCC patients (high miR-650/low LATS2 expression; low miR-650/low LATS2 expression; high miR-650/high LATS2 expression; low miR-650/high LATS2 expression) (**p<0.01).

Discussion

In this study, we demonstrated for the first time that miR-650 promotes HCC metastasis and EMT by directly targeting LATS2. MiR-650 reportedly helps mediate the progression of various cancers [8–10]. However, the role of miR-650 appears to differ depending on the type of cancer. Therefore, the effects and clinical significance of miR-650 in HCC as well as the underlying mechanisms should be further characterized. Our data from *in vivo* (clinical tissue samples) and *in vitro* analyses revealed novel information regarding the role of miR-650 in HCC progression.

miRNAs are commonly involved in the regulation of invasion and metastasis in various cancers [17, 18]. Most HCC patient deaths are due to recurrence and metastasis. Thus, we were interested in exploring whether miR-650 influences the progression of metastasis in HCC. Our examination of clinical tissue samples indicated that high miR-650 expression levels are associated with microscopic vascular invasion and the advanced TNM stage of HCC. Additionally, an *in vitro* assay involving Transwell plates revealed that miR-650 promotes HCC cell migration and invasion. There is accumulating evidence that miRNAs have a crucial function related to the progression of HCC EMT [19, 20]. Considering the EMT is a well-known basal mechanism related to cancer cell migration and invasion [3], we speculated that miR-650 may influence HCC EMT. Our western blot assay results suggested that decreased miR-650 levels increased epithelial marker expression and decreased mesenchymal marker expression, while increased miR-650 levels had the opposite effects. Moreover, an IHC assay involving HCC tissues further demonstrated that the miR-650 expression level is negatively associated with E-cadherin expression but is positively correlated with vimentin expression. Our results confirmed the previously reported aberrant expression of miR-650 in HCC tissues and its association with HCC progression [12]. Moreover, we further established the relationship between miR-650 and HCC EMT.

The Hippo signaling pathway regulates the biological behavior of multiple solid tumor cells [21]. Additionally, the function of the LATS2/YAP signal on the EMT of cancers, including HCC, has been described. In this study, we observed that *LATS2* is directly targeted by miR-650. A recent study involving NSCLC cells indicated that miR-650 promotes cell migration and invasion by targeting *LATS2* [22]. Our *in vitro* and *in vivo* analyses support these published findings, while also demonstrating that LATS2 counteracts the effects of miR-650 on HCC cell proliferation, migration, and invasion. Moreover, miR-650 inhibits *LATS2* expression, leading to the suppression of YAP and its downstream target genes. Therefore, miR-650 may promote HCC EMT by targeting *LATS2*. However, it is important to note that *LATS2* is not the only gene directly targeted by miR-650. A recent investigation concluded that miR-650 inhibits the progression of high-risk, non-metastatic colorectal cancer by suppressing the AKT2/GSK3β/E-cadherin pathway [23]. It was also suggested that miR-650 can decrease the sensitivity of lung adenocarcinoma cells to docetaxel by targeting *Bcl-2*/*Bax* [24]. It is unknown whether genes such as *AKT2* and *Bcl-2*/*Bax* are also targeted by miR-650 in HCC. Furthermore, among the genes targeted by miR-650, it is unclear whether *LATS2* is the most important determinant for the effects of miR-650 on HCC metastasis and EMT. The mechanisms underlying miR-650 functions related to HCC progression will need to be thoroughly investigated.

Many miRNAs may be relevant as diagnostic and prognostic biomarkers for HCC patients [25, 26]. We herein provide data for clinical samples obtained from 130 HCC patients, which revealed that high miR-650 levels and low LATS2 levels are associated with unfavorable clinical features and poor outcomes. These results may be useful for determining the prognosis for HCC patients. We also observed that miR-650 promotes HCC metastasis and EMT by directly inhibiting *LATS2* expression. Therefore, targeting the miR-650/*LATS2* pathway may represent a new therapeutic approach for HCC patients.

Conclusion

Our findings revealed the novel clinical and biological significance of miR-650 in HCC. Furthermore, the miR-650/*LATS2* pathway may be useful as a biomarker and potential therapeutic target for HCC patients.

Abbreviations

EMT (epithelial–mesenchymal transition); HCC (hepatocellular carcinoma); miRNA (MicroRNA); IHC (immunohistochemistry); GAPDH (glyceraldehyde 3-phosphate dehydrogenase); LATS2 (large tumor suppressor kinase 2); FBS (fetal bovine serum); YAP (yes-associated protein); OS (overall survival); DFS (disease-free survival).

Acknowledgements

Li li Han collected the clinical samples and performed most of the experiments. Xiao ran Yin performed RT-qPCR assay and the statistical analysis. Shu qun Zhang designed the study and assisted in the drafting of the manuscript. All authors have read and approved the final manuscript. The authors would like to thank Professor Chen Huang of Xi'an Jiaotong University (Xi'an, China) for providing the experimental platform and expert opinions.This study was supported by a grant from the Natural Science Basic Research Plan in Shaanxi Province of China (No. 1191329734) and China Postdoctoral Science General Financial Grant (no. 2017M623193).

Disclosure Statement

All authors declare they have no competing interests.

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