

Hypoxia-Induced Placenta-Specific microRNA (miR-512-3p) Promotes Hepatocellular Carcinoma Progression by Targeting Large Tumor Suppressor Kinase 2

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Background: Sustained proliferation and active metastasis are hallmarks of cancer, and they pose major challenges to the development of treatments and a cure for hepatocellular carcinoma (HCC). Thus, the mechanisms of proliferation, migration, and invasion of cancer cells need to be investigated. Many studies indicate that dysregulation of microRNA plays important roles in the progression of HCC, but the role of placenta-specific microRNA (miR-512-3p) in HCC has not been systematically investigated.

Purpose: In the current study, the expression, biological function, and mechanisms of miR-512-3p involvement in HCC were investigated.

Methods: Real-time quantitative polymerase chain reaction assays were conducted to determine miR-512-3p levels in HCC tissues and cell lines. The StarBase V3.0 online platform was used to compare miR-512-3p levels in HCC tissues with TCGA data and to identify potential miR-512-3p target genes. Associations between miR-512-3p and clinicopathological characteristics were analyzed statistically. MTT, ethynyl deoxyuridine, and transwell assays were performed to assess cell viability, proliferation, migration, and invasion. The luciferase reporter gene assay was used to verify target genes. Rescue assays were performed to confirm whether large tumor suppressor kinase 2 (LATS2) participated in the regulatory effects of miR-512-3p on HCC cell proliferation and motility, and whether miR-512-3p mediated the tumor-promoting effects of hypoxia.

Results: miR-512-3p was upregulated in HCC and it was associated with worse survival and unfavorable clinicopathological characteristics. Functional assays indicated that miR-512-3p contributed to HCC cell proliferation, migration, and invasion. Mechanistically, LATS2—a downstream target of miR-512-3p—mediated the tumor-promoting effects of miR-512-3p in HCC. Hypoxia could elevate miR-512-3p levels in HCC cells, and miR-512-3p partially mediated the tumor-promoting effects of hypoxia.

Conclusion: Hypoxia-induced miR-512-3p contributes to HCC cell proliferation, migration, and invasion by targeting LATS2 and inhibiting the Hippo/yes-associated protein 1 pathways.

Keywords: hepatocellular carcinoma, invasion, migration, miR-512-3p, LATS2, proliferation

Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver cancer, and it is a major cause of cancer-related deaths worldwide.^{1–3} Approximately half of the total number of HCC cases and deaths worldwide occur in residents of China, and this is partly associated with the comparative prevalence of hepatitis B in that

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country.^{4,5} Due to a lack of effective early diagnostic indicators HCC is often at an advanced stage when it is diagnosed, after the optimal treatment time has passed.⁶ Recurrence and metastasis after surgical resection are associated with a poor prognosis.⁷ It is thus essential to identify diagnostic indicators and to investigate the molecular mechanisms involved in HCC progression, in an effort to improve the accuracy of diagnosis and the efficacy of treatment.

Numerous studies indicate that dysregulated microRNAs are involved in the progression of HCC because they regulate the functioning of genes involved in various cellular processes including proliferation, invasion,^{8–11} and migration,^{12–14} and metastasis^{15,16} by regulating key genes. In recent years the involvement of placenta-specific microRNA (miR-512-3p) has been identified in various human cancers. Zhu et al¹⁷ reported that inhibition of dedicator of cytokinesis 3 (DOCK3) by miR-512-3p contributed to suppression of metastasis in non-small cell lung cancer. In several other studies miR-512-3p has been upregulated in HCC.^{18,19} To date however, no study has systematically investigated the role of miR-512-3p in HCC.

Hippo signaling evidently has an inhibitory effect on HCC progression.^{20,21} The Hippo signaling pathway activates large tumor suppressor kinases, which phosphorylate yes-associated protein 1 (YAP), resulting in cytoplasmic YAP retention. Previous studies indicate that large tumor suppressor kinase 2 (LATS2), a key component of the Hippo signaling pathway, functions as a tumor suppressor gene in various cancers including lung cancer,²² glioma,²³ endometrial cancer,^{24,25} colorectal cancer,²⁶ breast cancer,²⁷ esophageal squamous cell carcinoma,²⁸ and HCC.²⁹ LATS2 is regulated by several microRNAs. Xu et al³⁰ reported that microRNA-302d promotes the proliferation of human pluripotent stem cell-derived cardiomyocytes by inhibiting LATS2 in the Hippo pathway. Cheng et al²⁷ reported that miR-372 promotes breast cancer cell proliferation by directly targeting LATS2. Han et al³¹ reported that miR-103 promotes the metastasis and epithelial-mesenchymal transition of HCC by directly inhibiting LATS2. Notably however, relationships between LATS2 and miR-512-3p in HCC remain unknown.

Hypoxia is a main feature of HCC, and strong evidence suggests that it may promote HCC progression by regulating characteristics associated with malignancy, including cancer stem-like properties,³² proliferation,^{33,34} metastasis,^{35,36} and epithelial-mesenchymal transition^{37,38} in both hypoxia-inducible factor 1- α (HIF1- α)-

dependent and HIF1- α -independent manners. Dou et al³⁹ reported that hypoxia contributed to growth and metastasis by increasing the expression of tuftelin 1, which is an HCC oncoprotein. Zhou et al⁴⁰ reported that hypoxia induced glycolysis in HCC cells by increasing the expression of long non-coding RNA retinoic acid early transcript 1K pseudogene, mediated by HIF1- α . Zheng et al⁴¹ recently reported that hypoxia drove tumorigenesis and metastasis in HCC by downregulating miR-196-5p, but relationships between hypoxia and miR-512-3p in HCC remain uncharacterized.

In the present study miR-512-3p levels in HCC tissues and cells were investigated. A series of functional experiments was then performed to explore the biological roles of miR-512-3p in HCC cell proliferation and motility. The downstream target gene mediating the effects of miR-512-3p on HCC cell proliferation and motility was then screened for and verified. Lastly, the effects of hypoxia on miR-512-3p expression were investigated.

Materials and Methods

Tissue Samples

Tissue samples were obtained from 45 patients who underwent liver resection at the Department of General Surgery at the First Affiliated Hospital of Nanchang University (Nanchang, China). None of the patients received any adjuvant therapy such as chemotherapy or radiotherapy before surgery. All HCC and non-tumor tissues were stored in liquid nitrogen after they were collected. All patients provided written informed consent, and the study was approved by the Ethics Committee of Nanchang University, China. The clinicopathological parameters of the patients are shown in Table 1.

Cell Culture

The HEK293T cell line, L02 cell line (human immortalized normal hepatic cell line), and human HCC cell lines (Hep3B, SMMC-7721, MHCC97-L, and HCCLM3) were purchased from the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco, Grand Island, NY, USA) containing 1% penicillin-streptomycin (Sigma, St. Louis, MO, USA) and 10% fetal bovine serum (Gibco) in a 37°C incubator with 5% CO₂. The hypoxic cell model was generated by culturing cells in a 37°C hypoxia incubator with 1% O₂.

Table 1 Association Between miR-512-3p Expression and Clinicopathologic Features of Patients with Hepatocellular Carcinoma

Characteristics	Number (n=45)	miR-512-3p Levels		P-value
		High (n=23)	Low (n=22)	
Age (years)				0.292
<60	15	6	9	
≥60	30	17	13	
Gender				0.445
Male	33	18	15	
Female	12	5	7	
HBV infection				0.654
Negative	9	4	5	
Positive	36	19	17	
Liver cirrhosis				0.150
Absent	12	4	8	
Present	33	19	14	
AFP (ng/mL)				0.666
<20	11	5	6	
≥20	34	18	16	
Tumor size				0.026*
<5cm	21	7	14	
≥5cm	24	16	8	
Tumor multiplicity				0.608
Single	29	14	15	
Multiple	16	9	7	
Vascular invasion				0.042*
No	28	11	17	
Yes	17	12	5	
Edmondson–Steiner grade				0.399
I + II	30	14	16	
III+IV	15	9	6	
TNM stage				0.009*
I + II	33	13	20	
III+IV	12	10	2	

Note: *P<0.05, statistically significant difference.

Abbreviations: HBV, hepatitis B virus; AFP, alpha-fetoprotein; TNM, tumor-node-metastasis.

Cell Transfection

miR-512-3p mimics (miR-512-3p, miR10002823-1-5), control mimics (miR-control, miR1N0000001-1-5), miR-512-3p inhibitors (anti-miR-512-3p, miR20002823-1-5), and control inhibitors (anti-miR-NC, miR2N0000001-1-5) were purchased from RiboBio (Guangzhou, China). A LATS2 expression plasmid (LATS2, RC219394) and

a negative control (EV, PS100001) were purchased from OriGene Technologies Inc. (Rockville, MD, USA). The Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) was used in cell transfection assays, in accordance with the manufacturer's instructions.

Real-Time Quantitative Polymerase Chain Reaction

RNA was extracted from tissues and cells with TRIzol purchased from Invitrogen and the miRvana microRNA Isolation Kit in accordance with the manufacturer's instructions. Reverse transcription was then conducted using the TIANScript RT Kit (Tiangen Bio Inc., Beijing, China). Quantitative PCR was conducted with the SYBR Premix Ex Taq™ Kit (Takar Bio Inc., Kusatsu, Shiga, Japan) and TaqMan Human MiRNA Assay Kit (Genecopoeia Inc., Guangzhou, China). LATS2 and glyceraldehyde 3-phosphate dehydrogenase primers were purchased from Realgene (Nanjing, China). miR-512-3p and U6 primers were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). Expression levels were quantified via the $2^{-\Delta\Delta C_t}$ method. All primers used for quantitative real-time PCR (qRT-PCR) are shown in Table 2.

MTT Assay

MTT was purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA), and MTT assays were conducted to assess cell viability. Absorbance was read using a microplate reader (Bio-Rad, Hercules, CA, USA).

Ethynyl Deoxyuridine Incorporation Assay

Ethynyl deoxyuridine (EdU) incorporation assays were conducted to assess cell proliferation ability, using Cell-Light™

Table 2 Primers Used in This Study

Primers Name	Primer Sequence
LATS2 forward	5'- TGGCACCTACTCCCACAG-3'
LATS2 reverse	5'- CCAAGGGCTTCTTCATCT –3'
TXNIP forward	5'- GAGCCAGCCAACCTAAGAGA-3'
TXNIP reverse	5'- TAGCAGACACAGGTGCCATTA-3'
GAPDH forward	5'- GAAGGTGAAGGTCGGAGTC –3'
GAPDH reverse	5'- GAAGATGGTGATGGGATTTC –3'
miR-512-3p forward	5'- CGGCGGCACTCAGCCTTGAGGG –3'
miR-512-3p reverse	5'- GTGCAGGGTCCGAGGT –3'
U6 forward	5'- CTCGCTTCGGCAGCACA-3'
U6 reverse	5'- AACGCTTCACGAATTTGCGT –3'

EdU Apollo488 (RiboBio). A Zeiss fluorescence photomicroscope (Carl Zeiss, Oberkochen, Germany) was used to analyze the samples, and quantification was achieved by counting a minimum of five random fields per sample.

Transwell Assay

Transwell assays were conducted using transwell chambers (Millipore, Burlington, MA, USA), to assess the migration and invasion capacities of cells. In migration assays 3×10^4 HCC cells were cultured in the upper chamber with serum-free DMEM, and the lower chamber was filled with DMEM containing 20% serum. In invasion assays 3×10^4 HCC cells were seeded on Matrigel-coated membrane inserts, and the chamber was placed into a cell culture plate and incubated at 37°C for 24 h. Cells that had migrated or invaded across the transwell membrane were fixed in 4% paraformaldehyde for 30 min, then stained with 0.5% crystal violet for 30 min. A light microscope was used to analyze the samples, and quantification was achieved by counting a minimum of 10 random fields under 100x magnification.

Western Blotting

RIPA Buffer (WB009A; Hat Biotechnology, Xi'an, China) was used to extract proteins in HCC cells or tissues. A BCA kit (WB003; Hat Biotechnology) was used to measure protein concentrations. All proteins were then electrophoresed in a 10% sodium dodecyl sulfate polyacrylamide gel and transferred to polyvinylidene difluoride membrane (Bio-Rad). The membranes were blocked with 10% non-fat milk, then incubated with specific primary antibodies at 4°C overnight. The antibodies used in the study were anti-LATS2 (1:500; bs-4081R; Beijing Bioss Biotechnology), anti-YAP (1:1000; #14,074; Cell Signaling Technology, Danvers, MA, USA), anti-phospho-YAP (ser127; 1:1000; #13,008; Cell Signaling Technology), anti-thioredoxin-interacting protein (1:1000; #14,715; Cell Signaling Technology), and anti-glyceraldehyde 3-phosphate dehydrogenase (1:1000; #5174; Cell Signaling Technology). The membranes were then incubated with secondary antibody (anti-rabbit #7074 or anti-mouse #7076; Cell Signaling Technology) for 2 h at room temperature. Lastly, enhanced chemiluminescence reagent (PierceTM ECL, Thermo ScientificTM, Waltham, MA, USA) was applied to detect the proteins.

Luciferase Reporter Assay

Luciferase reporter assays were performed to confirm direct binding between the LATS2 3'-untranslated regions

(UTRs) and miR-512-3p. Wild-type (WT) and mutant (MUT) 3'UTRs of LATS2 mRNA were synthesized and inserted downstream of the promoter in the pEZX-MT06 vector (Genecopoeia). Cells transfected with miR-512-3p mimics, inhibitors, or corresponding control vectors were also transfected with LATS2-3'UTR-WT and LATS2-3'UTR-MUT. The cells were then incubated for 48 h. Lastly, the Luc-PairTM Duo-Luciferase Assay Kit (Genecopoeia) was used to quantify luciferase activity.

Statistical Analysis

Data are presented as means \pm the standard deviation, and at least three independent replicates were performed. One-way analysis of variance and two-tailed Student's *t*-test were performed using SPSS software 24.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 7.0 (San Diego, CA, USA). Statistical significance was assessed via the Kaplan–Meier method, Pearson's correlation analysis, and the Log rank test. Photoshop and Adobe Illustrator were used to generate images. $p < 0.05$ was deemed to indicate statistical significance.

Results

Clinical Outcomes and miR-512-3p in HCC

In HCC tissues miR-512-3p expression was higher than it was in non-tumor tissues harvested in the study ($p < 0.0001$, [Figure 1A](#)), and it was higher than that reported in the TCGA data pertaining to normal liver tissues accessed via the StarBase V3.0 online platform ($p = 0.00047$, [Figure 1B](#)). Higher miR-512-3p levels were observed in HCC cell lines (Hep3B, SMMC-7721, MHCC97-L, and HCCLM3) than in the immortalized normal liver cell line L02 ([Figure 1C](#)). In miR-512-3p-high and miR-512-3p-low groups of HCC patients generated based on median miR-512-3p expression, high miR-512-3p was significantly correlated with tumor size ($p = 0.026$), vascular invasion ($p = 0.042$), and advanced tumor-node-metastasis stage ($p = 0.009$) ([Table 1](#)). In Kaplan–Meier analysis HCC patients with high miR-512-3p expression exhibited worse overall survival ($p = 0.0115$, [Figure 1D](#)).

miR-512-3p and HCC Cell Proliferation, Migration, and Invasion

qRT-PCR results indicating the efficiency of transfection of Hep3B and HCCLM3 cells with miR-512-3p mimics and inhibitors are shown in [Supplementary Figure 1](#). In

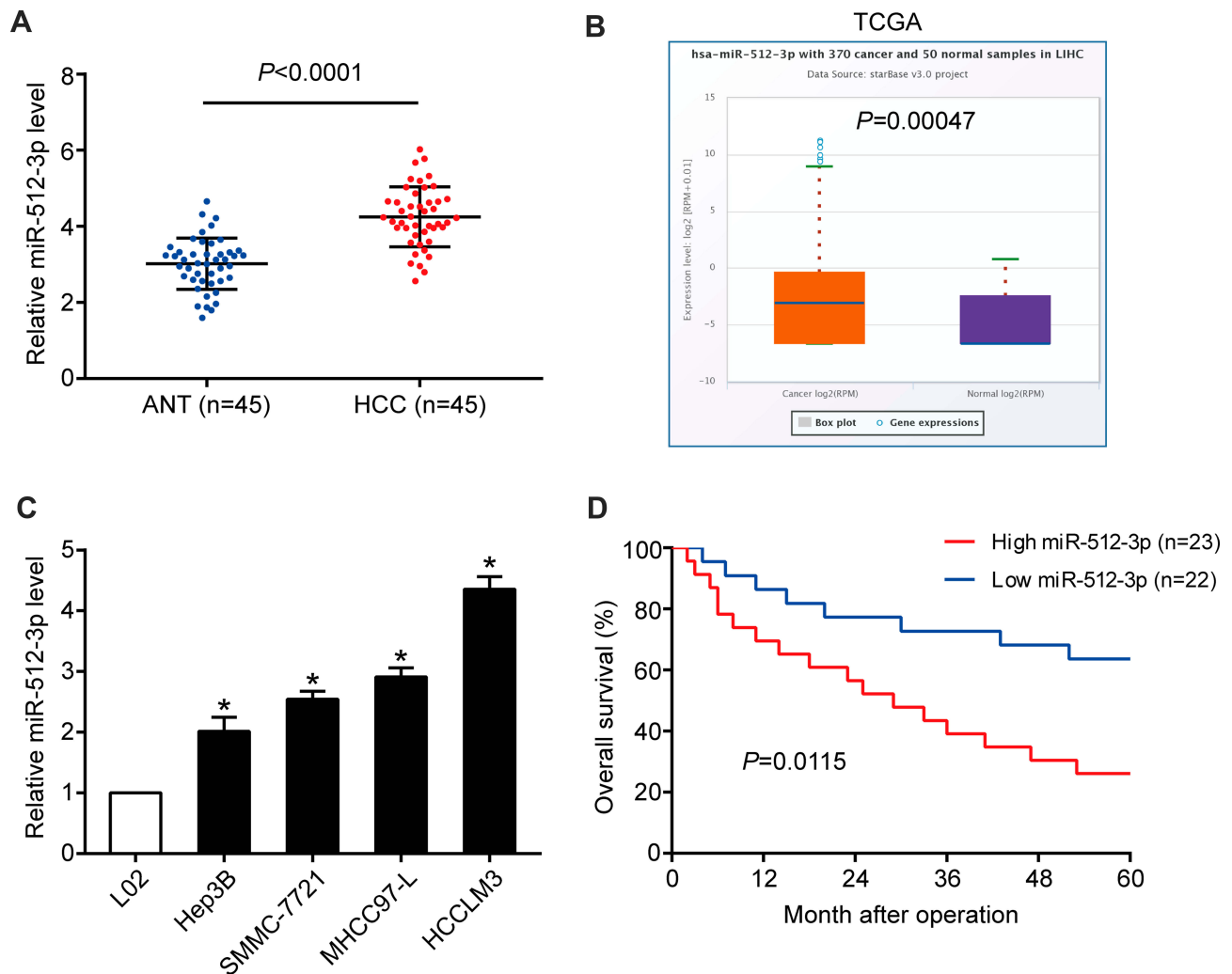


Figure 1 Expression and prognostic value of miR-512-3p in HCC. **(A)** miR-512-3p levels in 45 human HCC samples and 45 adjacent normal tissue samples ($p < 0.0001$, Student's *t*-test). **(B)** miR-512-3p levels were higher in the HCC tissues in the current study than the levels derived from normal liver tissues recorded in the TCGA database, accessed via the StarBase V3.0 platform ($p = 0.0005$, Student's *t*-test). **(C)** qRT-PCR was performed to determine miR-512-3p levels in HCC cell lines (Hep3B, SMMC-7721, MHCC97-L, and HCCLM3) and L02 cells. * $p < 0.05$, Student's *t*-test vs L02, $n = 3$. **(D)** In Kaplan-Meier survival analysis HCC patients with higher expression of miR-512-3p ($n = 23$) exhibited shorter overall survival than those with lower miR-512-3p expression ($n = 22$). $p = 0.0115$, Log rank test.

MTT and EdU assays miR-512-3p mimics significantly enhanced the viability and proliferation of Hep3B cells, whereas miR-512-3p inhibitors reduced the viability and proliferation of HCCLM3 cells ($p < 0.05$, Figure 2A–D). In transwell migration and invasion assays miR-512-3p mimics markedly increased the number of Hep3B cells that passed through the membrane ($p < 0.05$, Figure 2E), and the number of MHCC97-H cells that passed through the membrane was significantly reduced by miR-512-3p inhibitors ($p < 0.05$, Figure 2F).

miR-512-3p and LATS2 Targeting in HCC

In qRT-PCR and Western blot assays conducted using HCC cells, only LATS2 was significantly downregulated by miR-512-3p (Figure 3D–G and Supplementary Figure 2A and B).

Accordingly, LATS2 was selected as the target of miR-512-3p, and the complementary sequence between miR-512-3p and the 3'UTR of LATS2 is shown in Figure 3A. In qRT-PCR assays performed to detect LATS2 mRNA levels in 45 pairs of HCC tissues and adjacent non-tumor tissues, LATS2 expression was lower in HCC tissues ($p < 0.0001$, Figure 3B). miR-512-3p expression was inversely correlated with LATS2 mRNA levels in HCC tissues ($r = -0.7785$, $p < 0.0001$, Figure 3C). qPCR and Western blot assays conducted to assess LATS2 levels in Hep3B cells treated with miR-512-3p mimics and HCCLM3 cells treated with miR-512-3p inhibitors indicated that LATS2 was significantly negatively regulated by miR-512-3p at the mRNA level and the protein level ($p < 0.05$, Figure 3D–G). In luciferase reporter gene assays miR-512-3p overexpression

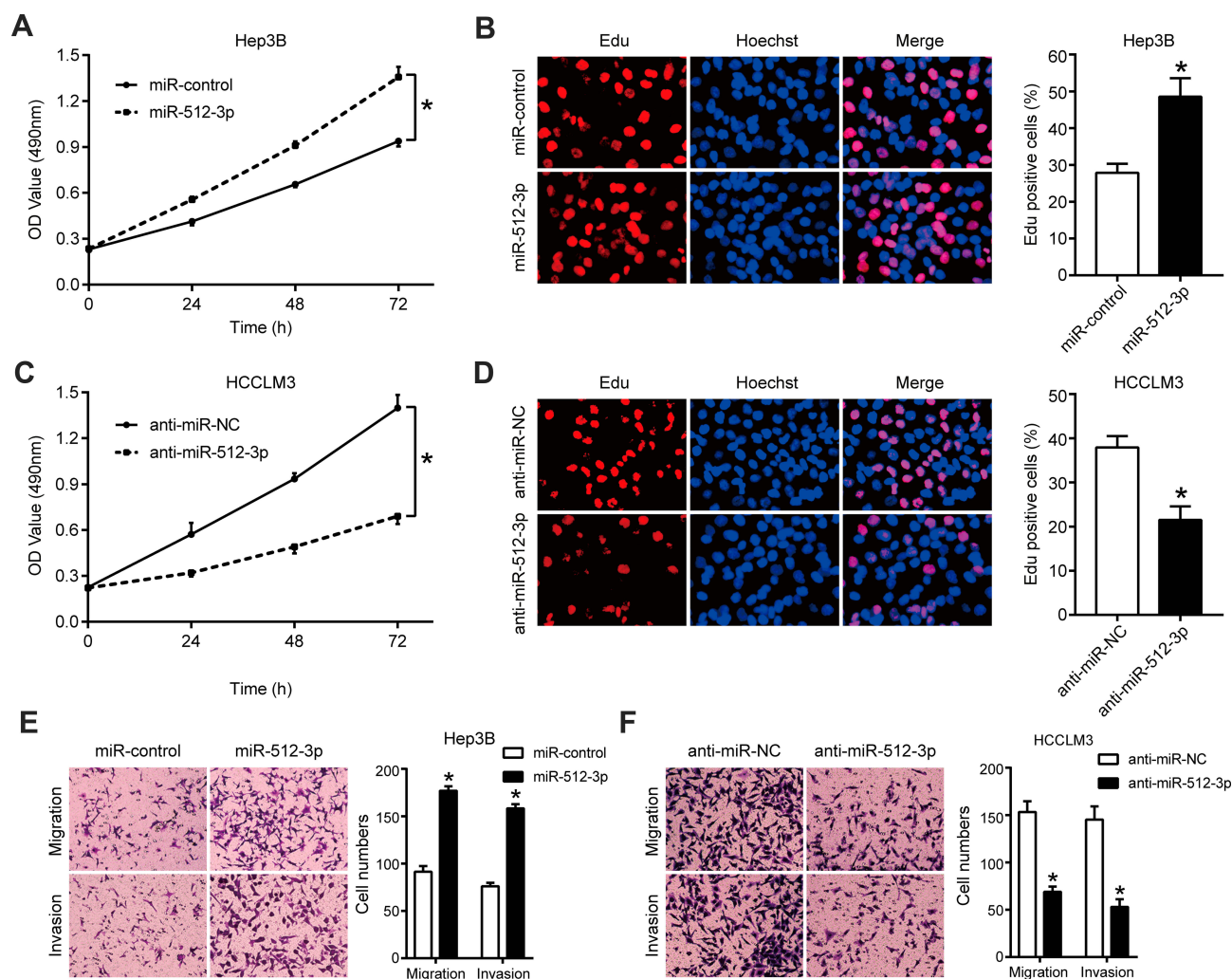


Figure 2 miR-512-3p promotes HCC cell proliferation and motility. **(A)** In MTT assays miR-512-3p mimics increased Hep3B cell viability. $*p < 0.05$, analysis of variance, $n = 3$. **(B)** In EdU assays miR-512-3p mimics enhanced Hep3B cell proliferation. $*p < 0.05$, Student's *t*-test; $n = 3$. **(C)** In MTT assays miR-512-3p inhibitors reduced HCCLM3 cell viability. $*p < 0.05$, analysis of variance, $n = 3$. **(D)** In EdU assays miR-512-3p inhibitors suppressed HCCLM3 cell proliferation. $*p < 0.05$, Student's *t*-test; $n = 3$. **(E)** In transwell migration and invasion assays miR-512-3p mimics enhanced the motility of Hep3B cells compared to control cells. $*p < 0.05$, Student's *t*-test, $n = 3$. **(F)** In transwell migration and invasion assays miR-512-3p inhibitors impaired the motility of HCCLM3 cells compared to control cells. $*p < 0.05$, Student's *t*-test; $n = 3$.

was suppressed but miR-512-3p knockdown enhanced the luciferase activity of the vector encoded with the WT-3'UTR of LATS2, but not the vector encoded with the MUT-3'UTR in HEK293T cells ($p < 0.05$, Figure 3H).

LATS2 Mediation of the Effects of miR-512-3p on Proliferation, Migration, and Invasion in HCC

LATS2 was overexpressed in Hep3B cells overexpressing miR-512-3p via transfection with a LATS2 expression plasmid, and the transfection efficiency as confirmed by Western blotting is shown in Figure 4A. The results of MTT and EdU assays indicated that

LATS2 overexpression partially abrogated the capacity of miR-512-3p to promote Hep3B cell viability and proliferation ($p < 0.05$, Figure 4B and C). In transwell assays the miR-512-3p mimic-induced enhanced motility of Hep3B cells was weakened after LATS2 overexpression ($p < 0.05$, Figure 4D).

Effects of miR-512-3p on Hippo/YAP Signaling Pathways in HCC

Western blot analysis indicated that miR-512-3p mimics reduced p-YAP expression, and that LATS2 overexpression partially abrogated the inhibitory effect of miR-512-3p mimics on p-YAP expression in Hep3B cells ($p < 0.05$, Figure 5).

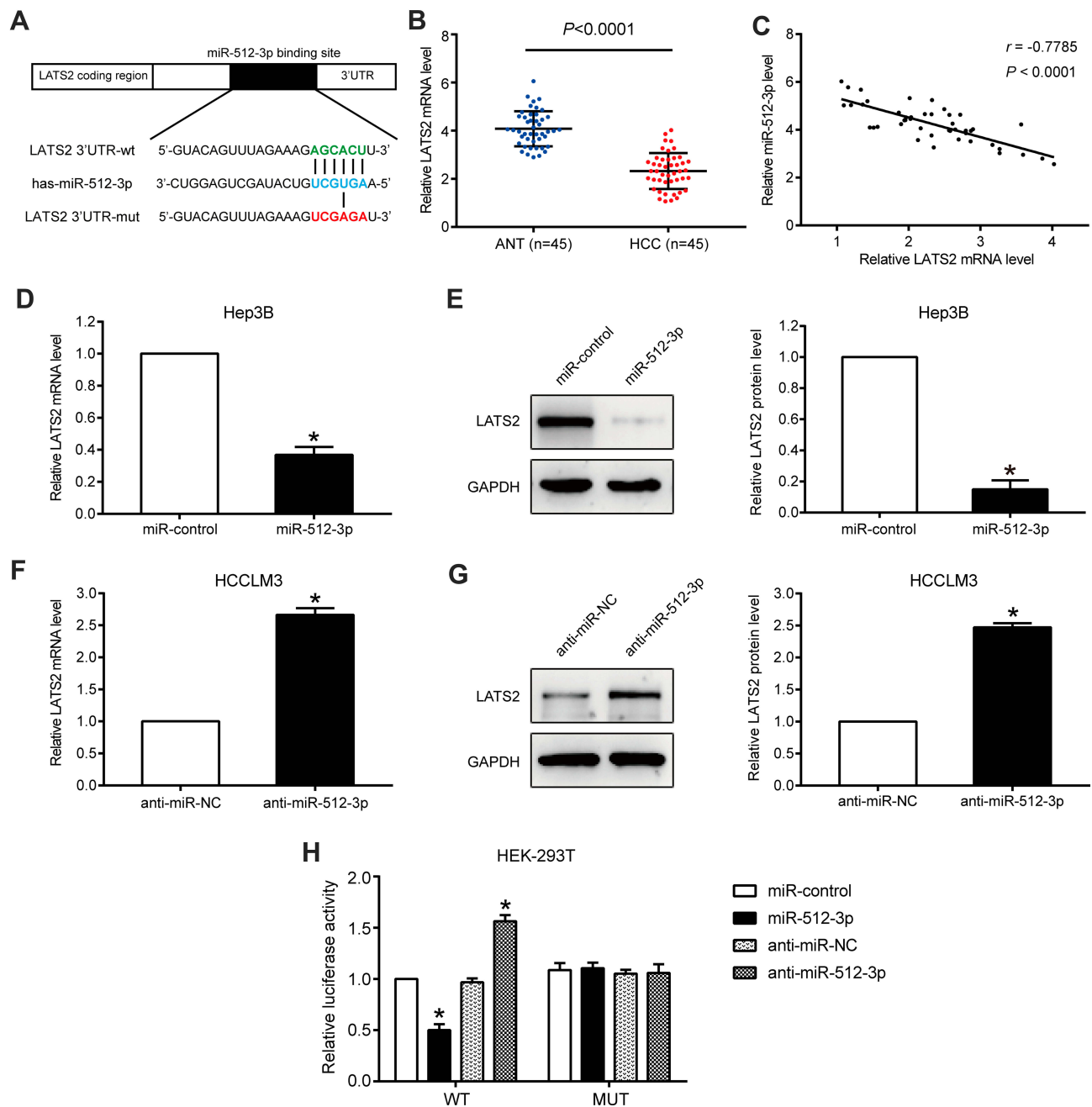


Figure 3 LATS2 is a direct target of miR-512-3p in HCC. (A) miR-512-3p and its putative binding sequence in the 3'UTR of LATS2. The MUT LATS2 binding site was generated in the complementary site for the seed region of miR-512-3p. (B) LATS2 mRNA levels in 45 HCC samples and 45 samples from adjacent normal tissue. $p < 0.0001$, Student's *t*-test. (C) In Pearson's correlational analysis there was a significant inverse correlation between LATS2 mRNA and miR-512-3p in HCC tissues ($r = -0.7785$, $p < 0.0001$). (D) In qRT-PCR analyses LATS2 was significantly downregulated by miR-512-3p at the mRNA level in Hep3B cells and (F) HCCLM3 cells. $*p < 0.05$, Student's *t*-test, $n = 3$. (E) In Western blot analysis LATS2 was significantly downregulated by miR-512-3p at the protein level in Hep3B cells and (G) HCCLM3 cells. $*p < 0.05$, Student's *t*-test, $n = 3$. (H) miR-512-3p overexpression significantly suppressed the luciferase activity of WT but not MUT 3'UTRs of LATS2. miR-512-3p knockdown caused a dramatic increase in luciferase activity of WT but not MUT 3'UTRs of LATS2 in HEK293T cells. $*p < 0.05$, Student's *t*-test, $n = 3$.

Hypoxia and miR-512-3p Expression in HCC

Hypoxia is an important feature of the microenvironment of solid tumors, and it promotes characteristics associated with malignancy such as growth^{39,42} and metastasis.^{43,44} In Hep3B cells cultured in hypoxic conditions (1% O₂) for

24 h HIF1- α protein was significantly upregulated ($p < 0.05$, Figure 6A), confirming that the hypoxic cell model had been successfully generated. Hypoxia markedly increased miR-512-3p levels ($p < 0.05$, Figure 6B) and inhibited LATS2 expression ($p < 0.05$, Figure 6A). In functional assays hypoxia promoted the viability, proliferation, and

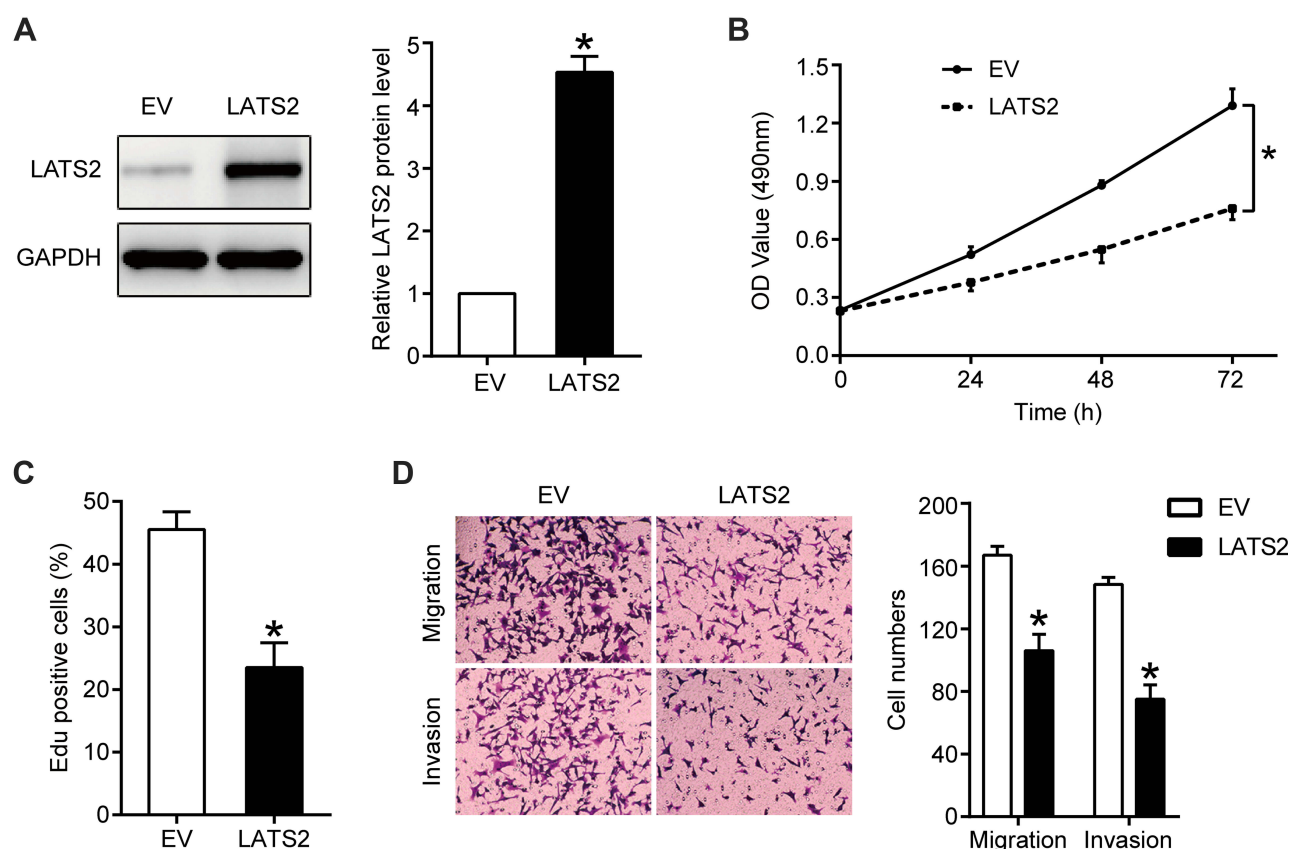


Figure 4 LATS2 overexpression partially abrogated the miR-512-3p overexpression-induced HCC cell proliferation, migration, and invasion. In Hep3B cells overexpressing miR-512-3p, LATS2 was overexpressed via transfection with a plasmid and the transfection efficiency was confirmed by Western blotting (A). * $p < 0.05$, Student's t -test, $n = 3$. (B) MTT, (C) EdU, and (D) transwell assays were performed to assess the viability, proliferation, migration, and invasion capacities of HCCLM3 cells transfected with the corresponding vectors. * $p < 0.05$, analysis of variance or Student's t -test, $n = 3$.

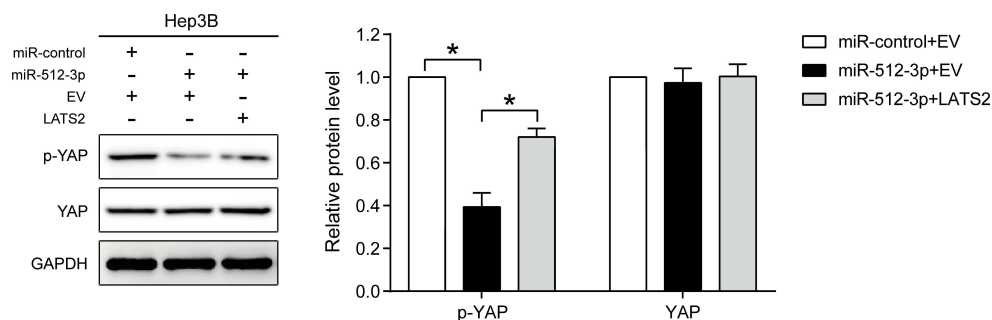


Figure 5 miR-512-3p inhibits the phosphorylation of YAP in HCC by targeting LATS2. Western blot indicating that miR-512-3p suppressed the phosphorylation of YAP in HCC, and that LATS2 overexpression reversed these effects. * $p < 0.05$, Student's t -test, $n = 3$.

mobility of Hep3B cells, and miR-512-3p inhibitors partially reversed the tumor-promoting effects of hypoxia ($p < 0.05$, Figure 6C–E).

Discussion

MicroRNAs are involved in the regulation of HCC progression.^{45,46} The roles of miR-512-3p have recently

been investigated in various tumors. Duan et al⁴⁷ reported that miR-512-3p regulated malignant tumor behavior and multi-drug resistance in breast cancer cells by targeting Livin. Zhu et al¹⁷ reported that inhibition of RAC1-GEF DOCK3 by miR-512-3p contributed to the suppression of metastasis in non-small cell lung cancer. Notably however, the biological function of miR-512-3p in HCC remains

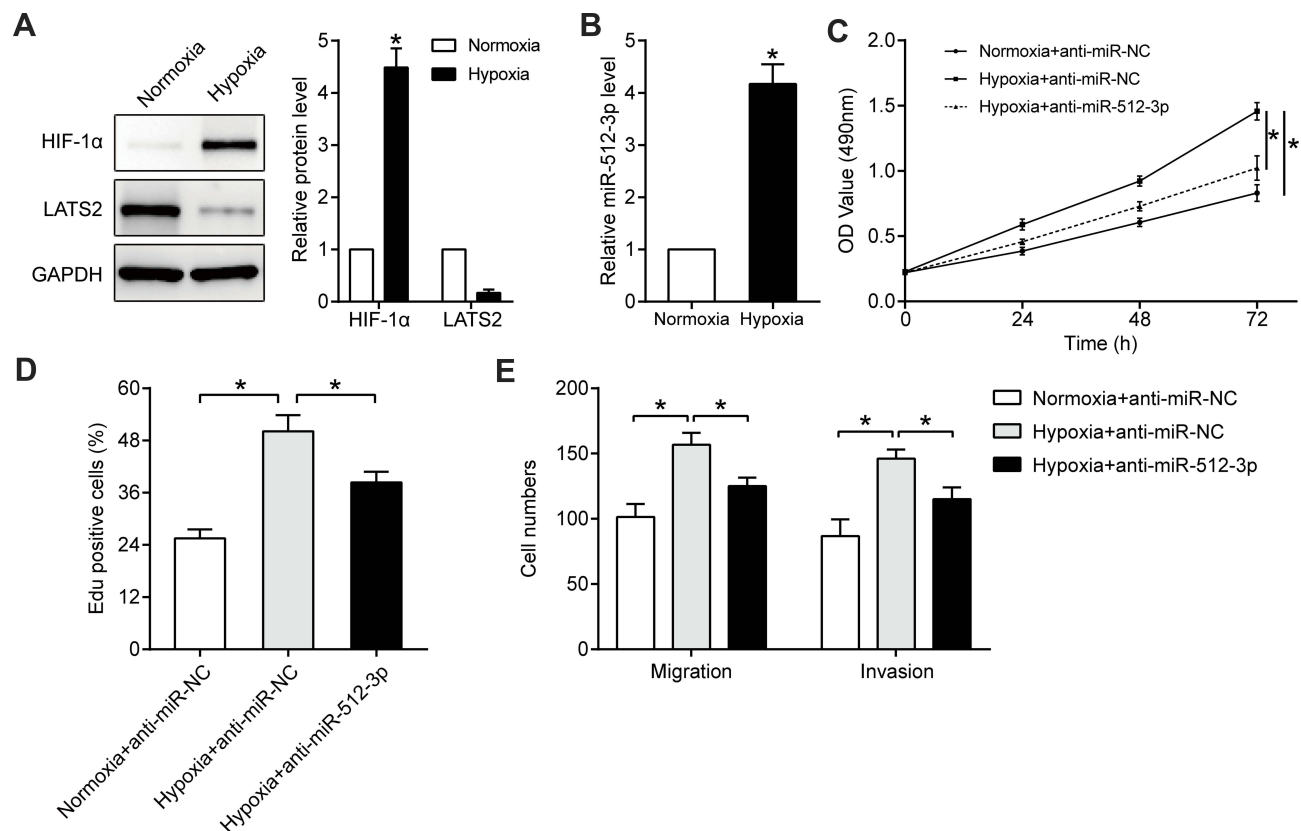


Figure 6 miR-512-3p mediates the tumor-promoting effects of hypoxia. Western blotting was performed to quantify HIF1- α and LATS2 levels in Hep3B cells cultured under normoxic or hypoxic conditions (A). * $p < 0.05$, Student's t -test, $n = 3$. (B) qRT-PCR was conducted to quantify miR-512-3p levels in Hep3B cells cultured under normoxic or hypoxic conditions. * $p < 0.05$, Student's t -test, $n = 3$. (C) MTT, (D) EdU, and (E) transwell assays were performed to assess the viability, proliferation, migration, and invasion capacities of Hep3B cells with or without miR-512-3p knockdown under hypoxic conditions. * $p < 0.05$, analysis of variance or Student's t -test, $n = 3$.

unclear. In the current study miR-512-3p was significantly elevated in HCC, and elevated miR-512-3p was associated with worse survival and unfavorable clinicopathological characteristics including tumor size, vascular invasion, and advanced tumor-node-metastasis stages. In functional experiments miR-512-3p promoted HCC cell proliferation and mobility. Collectively these results suggest that miR-512-3p functions as an oncogene in HCC.

In HCC Hippo signaling acts as a cancer suppression pathway by inhibiting tumor-related processes, including proliferation,⁴⁸ migration,⁴⁹ and invasion.⁵⁰ In the Hippo pathway LATS kinases phosphorylate YAP and cause the cytoplasmic retention and degradation of YAP. In previous studies various microRNAs have been linked to LATS2 regulation in human cancers, including miR-372 in breast cancer,²⁷ miR-492 in retinoblastoma,⁵¹ miR-135b in cutaneous melanoma,⁵² miR-363 in ovarian cancer,⁵³ and miR-103 in HCC.³¹ Relationships between LATS2 and miR-512-3p have not been investigated in HCC. The present study generated substantial evidence that LATS2 is a direct functional target of

miR-512-3p in HCC. miR-512-3p level was inversely correlated with LATS2 expression in HCC tissues. miR-512-3p downregulated LATS2 expression in HCC cells at the mRNA level and the protein level, and miR-512-3p affected the luciferase activity of the WT-3'UTR of LATS2 but not the MUT-3'UTR of LATS2. Lastly, in rescue assays restoration of LATS2 reversed the effects of miR-512-3p on HCC cell proliferation and mobility by activating the Hippo/YAP pathway. These results confirm that LATS2 is a direct functional target of miR-512-3p.

Hypoxia is an important feature of the microenvironment of solid tumors, and promotes malignancy.^{32–36,44} In several previous studies microRNAs have reportedly mediated the cancer-promoting effects of hypoxia in various tumor types.⁵⁴ Zheng et al⁴¹ suggested that hypoxia could drive tumorigenesis and metastasis in HCC by downregulating miR-196-5p. In the present study relationships between hypoxia and miR-512-3p expression were investigated for the first time. Hypoxia could upregulate miR-512-3p expression in HCC, and hypoxia-induced miR-512-3p partially mediated the tumor-promoting

effects of hypoxia. The mechanism involved in the elevated miR-512-3p expression induced by hypoxia in HCC will be investigated in future studies.

In summary, the current study suggests that miR-512-3p functions as an oncogene in HCC, and promotes the proliferation and mobility of HCC cells. Upregulated miR-512-3p expression was associated with reduced survival and unfavorable clinicopathological characteristics. Mechanistically miR-512-3p promotes tumorigenic characteristics by targeting LATS2, which results in reduced Hippo signaling. Hypoxia elevated miR-512-3p levels in HCC cells, and miR-512-3p partially mediated the tumor-promoting effects of hypoxia. Results of the present study suggest that miR-512-3p may be a novel therapeutic target for HCC treatment.

Abbreviations

HCC, hepatocellular carcinoma; HIF1- α , hypoxia-inducible factor 1-alpha; LATS2, large tumor suppressor kinase 2; miR-512-3p, placenta-specific microRNA; MUT, mutant; qRT-PCR, quantitative real-time PCR; UTR, untranslated region; WT, wild-type; YAP, yes-associated protein 1.

Acknowledgments

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Disclosure

The authors declare that there are no conflicts of interest pertaining to this work.

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