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

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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
country. 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. Recurrence and metastasis after surgical resection are associated with a poor prognosis. 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, and migration, and metastasis 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 al 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. 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. 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, glioma, endometrial cancer, colorectal cancer, breast cancer, esophageal squamous cell carcinoma, and HCC. LATS2 is regulated by several microRNAs. Xu et al reported that microRNA-302d promotes the proliferation of human pluripotent stem cell-derived cardiomyocytes by inhibiting LATS2 in the Hippo pathway. Cheng et al reported that miR-372 promotes breast cancer cell proliferation by directly targeting LATS2. Han et al 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 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.
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 TaqTM 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^−ΔΔCt 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 1 Association Between miR-512-3p Expression and Clinicopathologic Features of Patients with Hepatocellular Carcinoma |
|---------------------------------------------------------------|
| **Characteristics** | **Number** | **miR-512-3p Levels** | **P-value** |
| | | **High** (n=23) | **Low** (n=22) | |
| Age (years) | | | |
| <60 | 15 | 6 | 9 | 0.292 |
| ≥60 | 30 | 17 | 13 | |
| Gender | | | |
| Male | 33 | 18 | 15 | 0.445 |
| Female | 12 | 5 | 7 | |
| HBV infection | | | |
| Negative | 9 | 4 | 5 | 0.654 |
| Positive | 36 | 19 | 17 | |
| Liver cirrhosis | | | |
| Absent | 12 | 4 | 8 | 0.150 |
| Present | 33 | 19 | 14 | |
| AFP (ng/mL) | | | |
| <20 | 11 | 5 | 6 | 0.666 |
| ≥20 | 34 | 18 | 16 | |
| Tumor size | | | |
| <5cm | 21 | 7 | 14 | 0.026^* |
| ≥5cm | 24 | 16 | 8 | |
| Tumor multiplicity | | | |
| Single | 29 | 14 | 15 | 0.608 |
| Multiple | 16 | 9 | 7 | |
| Vascular invasion | | | |
| No | 28 | 11 | 17 | 0.042^* |
| Yes | 17 | 12 | 5 | |
| Edmondson–Steiner grade | | | |
| I+II | 30 | 14 | 16 | 0.399 |
| III+IV | 15 | 9 | 6 | |
| TNM stage | | | |
| I+II | 33 | 13 | 20 | 0.009^* |
| 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
EdU Apollo488 (RiboBio). A Zeiss fluorescence microscope (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 × 10⁴ 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 × 10⁴ 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 Bios Biotechnology), anti-YAP (1:1000; #14,074; Cell Signaling Technology, Danvers, MA, USA), anti-phospho-YAP (ser127; 1:1000; #14,074; Cell Signaling Technology), anti-thioredoxin-interacting protein (1:1000; #14,715; Cell Signaling Technology), anti-glyceroldehyde 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 (Pierce™ ECL, Thermo Scientific™, 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-Pair™ Duo-Luciferase Assay Kit (Genecopoeia) was used to quantify luciferase activity.

**Statistical Analysis**

Data are presented as means ± 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.
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
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 \((p < 0.05, \text{ Figure 3H})\).

**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 \((p < 0.05, \text{ Figure 4B and C})\). In transwell assays the miR-512-3p mimic-induced enhanced motility of Hep3B cells was weakened after LATS2 overexpression \((p < 0.05, \text{ Figure 4D})\).

**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 \((p < 0.05, \text{ Figure 5})\).
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 and metastasis. 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
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. The roles of miR-512-3p have recently been investigated in various tumors. Duan et al.\(^47\) reported that miR-512-3p regulated malignant tumor behavior and multi-drug resistance in breast cancer cells by targeting Livin. Zhu et al.\(^17\) 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
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, migration, and invasion. 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, miR-492 in retinoblastoma, miR-135b in cutaneous melanoma, miR-363 in ovarian cancer, and miR-103 in HCC. 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. In several previous studies microRNAs have reportedly mediated the cancer-promoting effects of hypoxia in various tumor types. Zheng et al 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 in HCC.

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.
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.

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

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