

MicroRNA-381 suppresses cell growth and invasion by targeting the liver receptor homolog-1 in hepatocellular carcinoma

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Abstract. MicroRNAs (miRs) have emerged as prospective tools for human cancer therapy, including hepatocellular carcinoma (HCC) therapy. Previous studies have suggested that miR-381 functions as oncogenic or tumor-suppressive miRs in other cancer types. However, the role of miR-381 in HCC remains unknown. The present study investigated the expression and functional role of miR-381 in HCC. miR-381 expression was significantly decreased in HCC tissues and cell lines. miR-381 overexpression significantly inhibited HCC cell proliferation and colony formation, induced G0/G1 cell cycle arrest and suppressed cell invasion. Conversely, suppression of miR-381 showed the opposite effect in HCC cells. Bioinformatics analysis and dual-luciferase reporter assay results showed that miR-381 directly targeted the 3'-untranslated region of liver receptor homolog-1 (LRH-1), and quantitative polymerase chain reaction and western blot analysis results showed that miR-381 negatively modulated LRH-1 expression. Data elucidated that miR-381 directly regulated HCC cell growth and invasion, as well as the Wnt signaling pathways, by targeting LRH-1. Clinical tissue detection data revealed an inverse correlation between miR-381 and LRH-1 expression in HCC tissues, further indicating the functional significance of miR-381-LRH-1 in regulating HCC tumorigenesis. The present study indicates that miR-381 may be a novel tumor suppressor that blocks HCC growth and invasion by targeting LRH-1. The results present novel insights into understanding the molecular mechanism underlying HCC

tumorigenesis and provide a future direction to the development of therapeutic interventions for HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors with increasing morbidity and mortality all over the world (1). Although current therapeutic strategies have improved over the past decades, the treatment outcome and survival rate remain poor because of frequent recurrence and high invasion and metastasis capacity (2-4). One of the obstacles that hampers the development of efficient therapies is the lack of understanding of the precise molecular mechanism underlying HCC development and progression. Thus, a better understanding of the pathological mechanism is important to provide theoretical basis for developing potential and promising therapeutics for HCC.

In recent years, a class of small and non-coding RNAs called microRNAs (miRs) have emerged as prospective tools for cancer therapy, including HCC therapy (5). miRs are negative regulators of gene expression that regulate protein translation by targeting the 3'-untranslated region (UTR) of mRNA (6,7). Studies have indicated that miRs act as potential and useful biomarkers and targets for disease diagnosis, prognosis and treatment (8,9). Other studies have also reported that miRs such as miR-302b (10), miR-1285-3p (11), miR-520g (12) and miR-139 (13) serve as oncogenes or tumor suppressors in HCC. miR-381 is extensively associated with various cancer types (14,15), but its role in HCC has not been comprehensively investigated.

The liver receptor homolog-1 (LRH-1) has been reported as an important regulator in many different cancer types (16). Various studies have uncovered that LRH-1 is involved in regulating embryonic development, ovulation, pregnancy and metabolism (17-22). LRH-1 deficiency blunts intestinal tumorigenesis (23). LRH-1 is elucidated as an oncogene in pancreatic cancer (24), colon cancer (25), gastric cancer (26), breast cancer (27) and ovarian cancer (28). Moreover, LRH-1 is associated with the activation of Wnt signaling pathways (29), which were frequently aberrantly activated in cancers, such as HCC (30). These findings indicate that LRH-1 can serve as a potential molecular target for cancer therapy. Several studies have also attempted to develop an effective antagonist for LRH-1 to treat cancers (31).

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Abbreviations: miRs, microRNAs; miR-381, microRNA-381; LRH-1, liver receptor homolog-1; 3'-UTR, 3'-untranslated region; HCC, hepatocellular carcinoma

Key words: hepatocellular carcinoma, miR-381, liver receptor homolog-1, cell growth, cell invasion

The suppression of LRH-1 in HCC has been demonstrated to induce cell cycle arrest and apoptosis (32), thus indicating its important role in HCC. However, the precise role of LRH-1 in HCC remains largely unknown. In the present report, we demonstrate a targeted relationship between LRH-1 and miR-381. We found that miR-381 was significantly downregulated in HCC cells and tissues. miR-381 overexpression inhibited HCC cell growth and invasion ability, whereas miR-381 suppression displayed an opposite effect. Bioinformatics analysis and dual-luciferase reporter assay results showed that miR-381 directly targeted the 3'-UTR of LRH-1, and quantitative polymerase chain reaction (qPCR) and western blot analysis results showed that miR-381 negatively modulated LRH-1 expression. Data elucidated that miR-381 regulated HCC cell growth and invasion through LRH-1-mediated Wnt signaling. Clinical tissue detection data revealed that an inverse correlation existed between miR-381 and LRH-1 expression, thus further indicating the functional significance of miR-381-LRH-1 in regulating HCC tumorigenesis. The present study provides evidence that the use of miR-381 to target LRH-1 may be a potential therapeutic strategy for HCC treatment.

Materials and methods

Tissue specimens. A total of 20 paired malignant HCC and adjacent non-tumorous liver tissues were obtained from HCC patients undergoing surgical resection in the First Hospital of Jilin University. These patients were first diagnosed without local or systemic treatment before surgical resection. The tissue samples were removed and immediately snap-frozen in liquid nitrogen for further use. Informed consent was obtained from each patient, and the experimental protocol was reviewed and approved by the Institutional Human Experiment and Ethic Committee of the First Hospital of Jilin University.

Cell cultures. Human HCC cell lines including HepG2, SMMC-7721, HuH7, BEL-7402, human normal liver cell line HL-7702 and human embryonic kidney (HEK) 293T cell line were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and were grown in Dulbecco's modified Eagle's medium (DMEM) in a supplement with 10% heat-inactivated fetal bovine serum (FBS) supplemented with 1% penicillin-streptomycin (all from Gibco, Grand Island, NY, USA). The cells were cultured in a humidified incubator containing 5% CO₂ atmosphere at 37°C.

RNA isolation and quantitative polymerase chain reaction (qPCR). Total RNA was extracted by using the miRNeasy Mini kit (Qiagen, Dusseldorf, Germany) according to the manufacturer's recommended method. For mRNA expression analysis, the first-strand cDNA was synthesised by using Moloney murine leukaemia virus (M-MLV) reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The cDNA was then used as templates to amplify LRH-1, cyclin D1, cyclin E1 and MMP9 with specific primers by using SYBR Green Master Mix (Bio-Rad, Hercules, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal normalisation control. For miRNA expression analysis, the cDNA was obtained using miScript reverse transcription kit and amplified using miScript SYBR Green PCR kit (both

from Qiagen) according to the manufacturer's instructions. U6 small nuclear RNA (U6) was used as internal normalization control. The specific primers used in this study were as follows: LRH-1 (forward, 5'-CTGATAGTGGAACTTT TGAA-3' and reverse, 5'-CTTCATTTGGTCATCAACCTT-3'); cyclin D1 (forward, 5'-GTTCGTGGCCTCTAAGATGAAG-3' and reverse, 5'-GTGTTTGGCGGATGATCTGTTTG-3'); cyclin E1 (forward, 5'-GCTTCGGCCTTGTATCATTTTC-3' and reverse, 5'-CTGTCTCTGTGGGTCTGTATG-3'); MMP9 (forward, 5'-GCCCGACCCGAGCTGACTC-3' and reverse, 5'-TTCAG GCGAGGACCATAGAGG-3'); GAPDH (forward, 5'-GAAA TCCATCACCATCTTCCAGG-3' and reverse, 5'-GAGCCC CAGCCTTCTCCATG-3'); miR-381 (forward, 5'-TAATCTGA CTATAAAGGGCAAGCT-3' and reverse, 5'-TATGGTTGT TCTGCTCTGTCTC-3'); and U6 (forward, 5'-GCTTCGGC AGCACATATACTAAAAT-3' and reverse, 5'-CGCTTCACGA ATTTGCGTGTTCAT-3'). Relative gene expression was analyzed by using the 2^{-ΔΔC_t} method.

Transfection of HCC cell lines. miR-381 mimics (sense, 5'-UAUACAAGGGCAAGCUCUCUGUTT-3' and antisense, 5'-ACAGAGAGCUUGCCCUUGUCGCTT-3'), scrambled mimics (sense, 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense, 5'-ACGUGACACGUUCGGAGAATT-3'), miR-381 LNA oligonucleotide (inhibitor; 5'-LNAACAGAGAGCUUG CCCUUGUAUA-3') and scrambled LNA (5'-LNACAGUAC UUUUGUGUAGUACAA-3') were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). Cells were plated onto 6-well plates and cultured until reaching 80% confluence. At that time, cells were transiently transfected with miRNA mimics, miR-LNA or their scrambled controls for a final concentration of 20 nM using Lipofectamine 2000 (Invitrogen) according to the supplier's recommended protocol. For LRH-1 overexpression, 4 μg of pcDNA3.0/LRH-1 expressing vectors were transiently transfected into HCC cells using Lipofectamine 2000 (Invitrogen) for 48 h. The transfection efficiency was then detected by using western blot analysis.

MTT assay. Cell growth was evaluated by using the MTT method. The HCC cells were briefly seeded in 96-well plates at 1x10⁴ cells/well and cultured overnight. The cells were then transfected with miRNA-381 mimics, miR-381 LNA or their scrambled controls and were incubated for 24, 48 and 72 h followed by discarding the old medium and adding fresh medium containing MTT (5 mg/ml in PBS; Sigma, St. Louis, MO, USA). After culturing for another 4 h at 37°C, the formed formazan products were solubilised by adding dimethyl sulfoxide (200 μl/well). The optical density (OD) value was detected at a wavelength of 490 nm by using a multi-well spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA).

Cell cycle analysis. Cell cycle distribution was detected by using flow cytometry. HCC cells were briefly transfected with miRNA-381 mimics or miR-381 LNA for 48 h. Cells were harvested by trypsinization, washed with PBS and fixed with 70% ethanol overnight at 4°C. Approximately 1x10⁶ cells were stained with propidium iodide (PI, 100 μg/ml; Sigma) solution containing 10 μg/ml of RNase A for 30 min in the dark. The percentage of cells in each cell cycle phase was then measured by using a FACScan flow cytometer (Becton

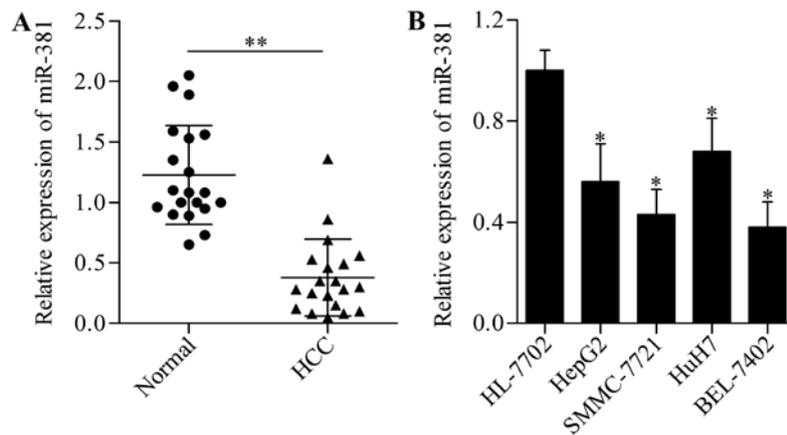


Figure 1. Decreased miR-381 expression in HCC. qPCR analysis of miR-381 expression in HCC tissues (A) and HCC cell lines (B). Non-cancerous counterparts and human normal liver cell line HL-7702 were used as control. ** $P < 0.01$ vs. normal; * $P < 0.05$ vs. HL-7702.

Dickinson, Franklin Lakes, NJ, USA) and analyzed by using ModFit software.

Colony formation assay. HCC cells were cultured in a 6-well plate and transfected with miRNA-381 mimics or miR-381 LNA for 48 h. Thereafter, the transfected cells were re-plated into a 6-well plate in a growth medium containing 0.3% noble agar at 200 cells/well to form natural colonies. After 2 weeks, cells were washed with PBS, fixed with 4% paraformaldehyde and stained with Giemsa (Sigma). The total number of colonies was counted under a microscope.

Cell invasion assay. HCC cells were transfected with miRNA-381 mimics or miR-381 LNA for 48 h and then starved overnight in serum-free medium. Thereafter, the transfected cells (1×10^5 cells) were seeded into the upper chamber of a 24-well Transwell (Corning Incorporated, Toledo, NY, USA) containing 200 μ l of serum-free medium. Transwell membrane filter inserts were precoated with Matrigel (BD Biosciences, San Jose, CA, USA). A medium containing 10% FBS added in the lower chamber was used as a chemoattractant. After incubation for 24 h, the non-invasive cells in the upper chamber were gently removed by a cotton swab. The invasive cells on the lower surface were then fixed, stained and observed under the microscope. The mean number of invaded cells in 5 random fields per membrane was counted and averaged.

Luciferase reporter assay. The targeting relationship between miR-381 and LRH-1 3'-UTR was detected by using a dual-luciferase reporter assay. The cDNA fragments from the 3'-UTR of LRH-1 containing miR-381 binding site were briefly amplified and subcloned into pmirGLO vector (Promega, Madison, WI, USA). HEK293T cells were seeded into 6-well plates at 2×10^5 cells/well for 24 h and then co-transfected with the 10 ng of pmirGLO-LRH-1 recombinant vectors and 20 nM of miR-381 mimics or scrambled mimics by using Lipofectamine 2000 (Invitrogen). Cells were lysed after 48 h of transfection, and the luciferase activity was measured by using the dual-luciferase reporter system (Promega). Wnt signaling activity was determined by using Tcf luciferase assays. Cells were briefly co-transfected with TOPFlash firefly luciferase reporter vector (100 ng; Addgene, Cambridge, MA,

USA) and pRL-TK *Renilla* luciferase vectors (5 ng; Promega) in the presence of miRNA-381 mimics or miR-381 LNA for 48 h. Cells were lysed, and firefly and *Renilla* luciferase activities were detected by using the dual-luciferase reporter system (Promega). The data were expressed as firefly/*Renilla* luciferase activity.

Western blot analysis. Cells were lysed by using RIPA lysis buffer, and protein concentrations were measured by using a BCA kit (both from Beyotime Biotechnology, Haimen, China). A total of 25 μ g proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and then electro-transferred onto a nitrocellulose membrane (Bio-Rad). The membrane was then blocked by 3% fat-free milk and blotted with primary antibodies (rabbit polyclonal anti-LRH-1, 1:500; rabbit polyclonal anti-GAPDH, 1:800; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 4°C overnight. Thereafter, the membrane was incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1:2,000; Santa Cruz Biotechnology Inc.) for 1 h at room temperature. The signals of the protein bands were detected by using the enhanced chemiluminescence method (Amersham, Little Chalfont, UK). The signal intensity on the membrane was analysed by Image-Pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA) and normalized to the control group.

Data analysis. All data were reported as means \pm standard deviation. Statistical analyses were performed with SPSS version 11.5 (SPSS Inc., Chicago, IL, USA) using Student's t-test or one-way analysis of variance. Correlations were assessed by Pearson's correlation test. A p-value of < 0.05 was set as the level of statistical difference.

Results

miR-381 downregulation frequently occurred in HCC tissues and cell lines. To investigate the potential function of miR-381 in HCC, we detected first the expression pattern of miR-381 in 20 paired malignant HCC and adjacent non-tumorous liver tissues by using qPCR. The results showed that miR-381 was frequently and significantly decreased in live HCC samples compared with their peritumor counterparts (Fig. 1A).

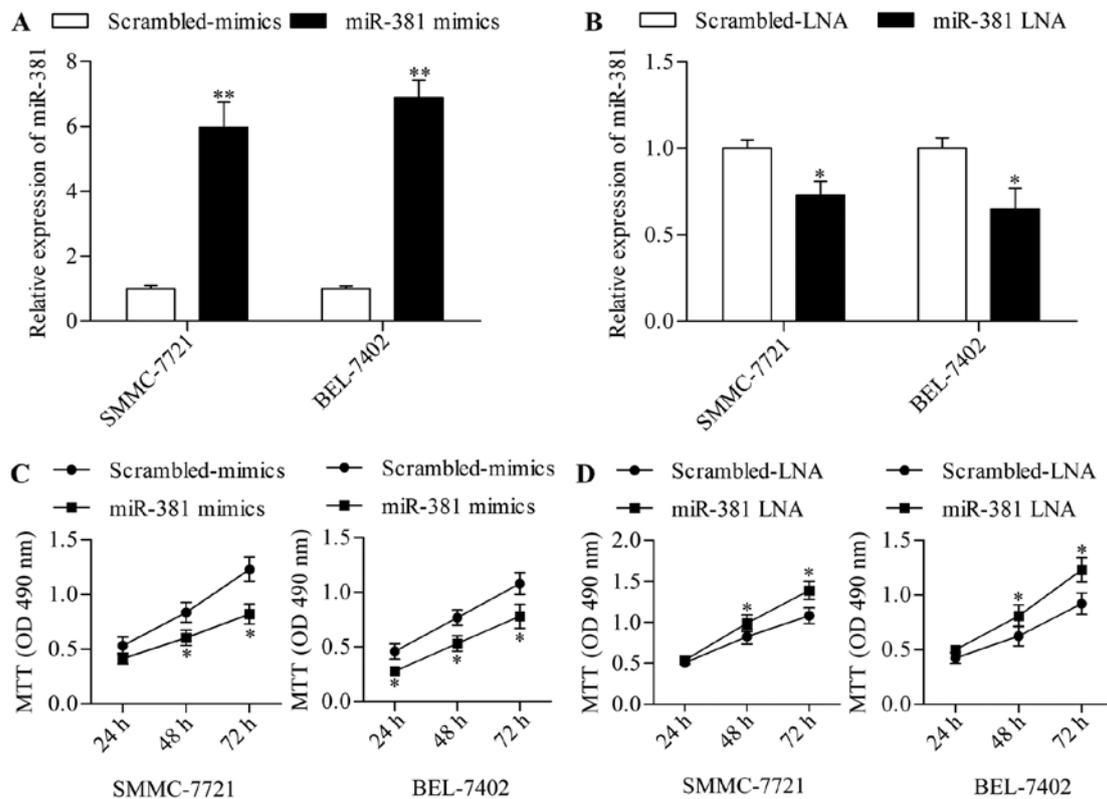


Figure 2. Detection of the role of miR-381 on HCC cell growth. qPCR analysis of miR-381 expression in HCC cells transfected with miR-381 mimics (A) and miR-381 LNA (B) or their scrambled controls. Cells were harvested for qPCR analysis after transfection for 48 h. ** $P < 0.01$ vs. scrambled mimics; * $P < 0.05$ vs. scrambled LNA. MTT assay detected the effect of miR-381 mimics (C) and miR-381 LNA (D) transfection on HCC cell growth at 24, 48 and 72 h. * $P < 0.05$ vs. scrambled mimics or scrambled LNA.

Furthermore, we detected the expression levels of miR-381 in several HCC cell lines (HepG2, SMMC-7721, HuH7 and BEL-7402) and normal liver cells. This observation was consistent with the results of miR-381 in HCC tissues wherein miR-381 expression was prevalently downregulated compared with human normal liver HL-7702 cells (Fig. 1B). These results indicated that miR-381 may play an important role in HCC development and progression.

miR-381 inhibits HCC cell growth. To explore the precise function of miR-381 in HCC, we next assessed the effect of miR-381 on HCC growth by using a gain-of-function approach with miR-381 mimics. SMMC-7721 and BEL-7402 cells were transfected with miR-381 mimics followed by detection using MTT assay. The results showed that miR-381 overexpression (Fig. 2A) significantly decreased HCC cell growth (Fig. 2C) in SMMC-7721 and BEL-7402 cells. By contrast, miR-381 suppression (Fig. 2B) significantly promoted HCC cell growth (Fig. 2D). To further verify its effect on cell growth, we examined the effect of miR-381 overexpression on cell cycle progression and found that miR-381 overexpression induced an increase in cell cycle arrest in G0/G1 phase (Fig. 3A). Moreover, the colony-forming capacity of HCC cells was remarkably suppressed by miR-381 overexpression (Fig. 3C). miR-381 suppression by transfection of miR-381 LNA conversely exhibited the opposite effect on cell cycle (Fig. 3B) and colony-forming capacity (Fig. 3D). These results implied that miR-381 functioned as a potential tumor suppressor in HCC.

miR-381 suppresses HCC cell invasion in vitro. To further verify the antitumor effect of miR-381 on HCC, we next examined the effect of miR-381 overexpression or suppression on HCC cell invasion by using Transwell invasion assay. The results showed that miR-381 overexpression significantly decreased the invasion of HCC cells (Fig. 4A), whereas miR-381 suppression further promoted this ability (Fig. 4B).

miR-381 directly targets and mediates LRH-1 expression. To uncover the molecular basis of miR-381 in regulating HCC, we sought to screen the putative targets of miR-381 by using bioinformatics analysis. Notably, among those targets, LRH-1 attracted our particular interests because of its important role in various cancers (16). As shown in Fig. 5A, the predicted complementary site of miR-381 was found in the 3'-UTR of LRH-1. We evaluated the dual-luciferase reporter activity by using pmirGLO vector harbouring the wild-type (WT) or mutant (MT) 3'-UTR of LRH-1 to verify whether LRH-1 was the direct target gene of miR-381. Luciferase assay displayed that miR-381 mimics significantly decreased the luciferase activity of pmirGLO-WT LRH-1, whereas miR-381 LNA markedly increased this activity (Fig. 5B). However, neither miR-381 mimics nor miR-381 LNA showed any effects on the luciferase activity of pmirGLO-MT LRH-1 (Fig. 5B). The data indicated that miR-381 directly targeted the predicted complementary site in the 3'-UTR of LRH-1. To further investigate whether miR-381 could regulate endogenous LRH-1 expression, we transfected miR-381 mimics or miR-381 LNA into SMMC-7721 cells and detected LRH-1 expression by using

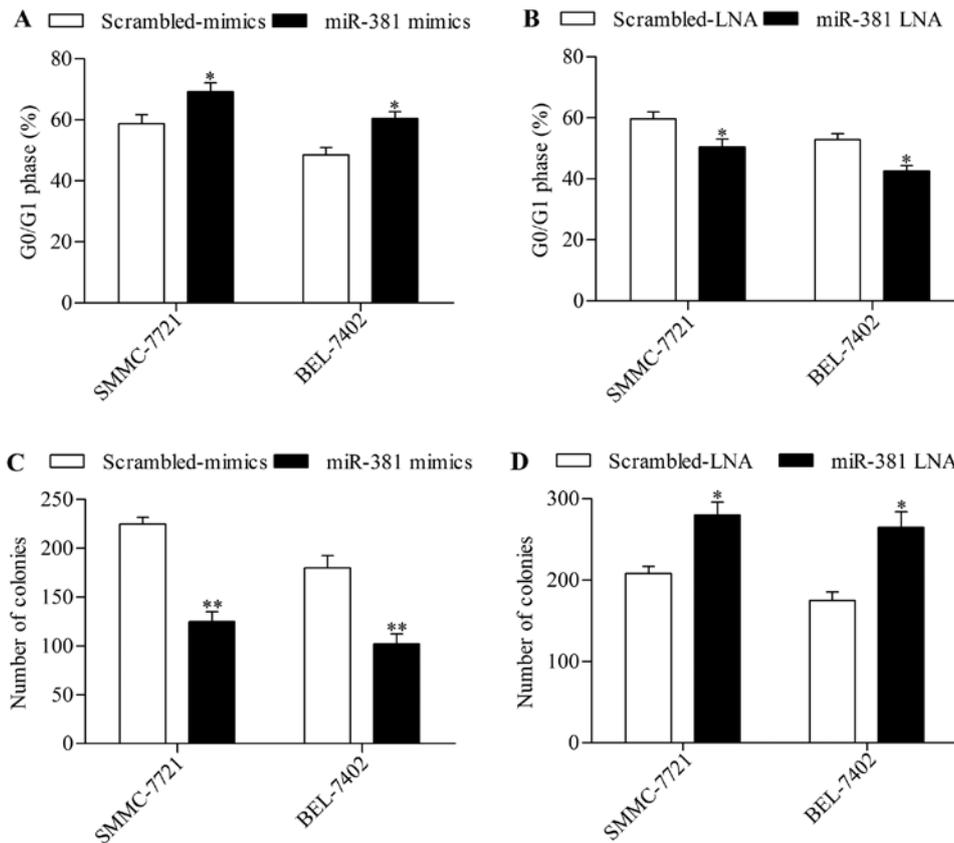


Figure 3. Detection of the role of miR-381 on HCC cell proliferation. The effect of miR-381 mimics (A) and miR-381 LNA (B) on cell cycle distribution was detected by using a cytometry assay. Cells were stained with PI fluorescent dye for flow cytometry assay after transfection for 48 h. *P<0.05 vs. scrambled mimics or scrambled LNA. Effect of miR-381 mimics (C) and miR-381 LNA (D) transfection on the colony-forming capacity of HCC cells. The numbers of colonies were counted by using the Giemsa staining method. **P<0.01 vs. scrambled mimics; *P<0.05 vs. scrambled LNA.

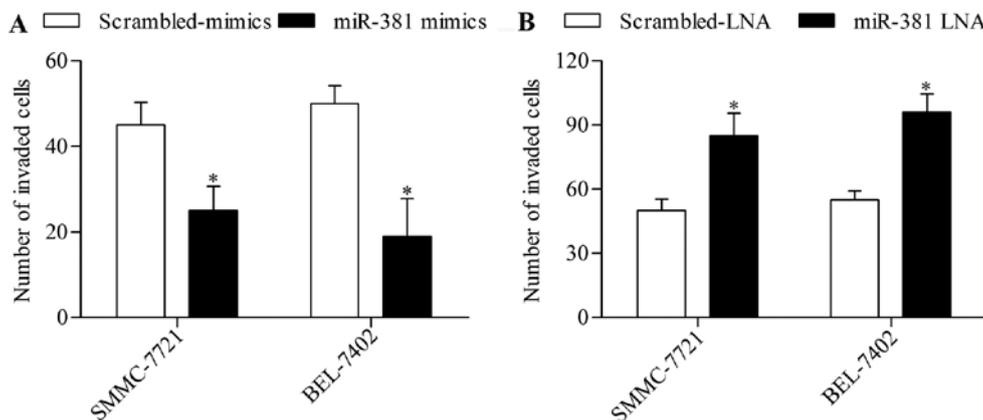


Figure 4. Effect of miR-381 on HCC cell invasion. The effect of miR-381 overexpression (A) or miR-381 suppression (B) on HCC cell invasion ability detected by using Transwell invasion assay. SMMC-7721 and BEL-7402 cells transfected with miR-381 mimics or miR-381 LNA were subjected to Transwell invasion assay. The invaded cells were counted by using the crystal violet staining method after 24 h. *P<0.05 vs. scrambled mimics or scrambled LNA.

qPCR and western blot analysis. The results showed that both mRNA and protein expression of LRH-1 was significantly decreased by miR-381 overexpression (Fig. 5C and D). On the contrary, miR-381 suppression further increased LRH-1 expression in SMMC-7721 cells (Fig. 5C and D). Consistent results were obtained by using BEL-7402 cells (data not shown).

miR-381 functions as a tumor suppressor to repress HCC cell growth and invasion through LRH-1. We performed a

rescue experiment to validate miR-381 functions through LRH-1. HCC cells were co-transfected with miR-381 mimics and LRH-1 expressing vectors without 3'-UTR. Western blot analysis showed that LRH-1 protein expression was significantly restored by LRH-1 expressing vectors transfection even though with miR-381 mimics transfection in SMMC-7721 (Fig. 6A) and BEL-7402 (Fig. 6B) cells. MTT assay showed that LRH-1 overexpression significantly reversed the inhibitory effect of miR-381 on HCC cell growth in SMMC-

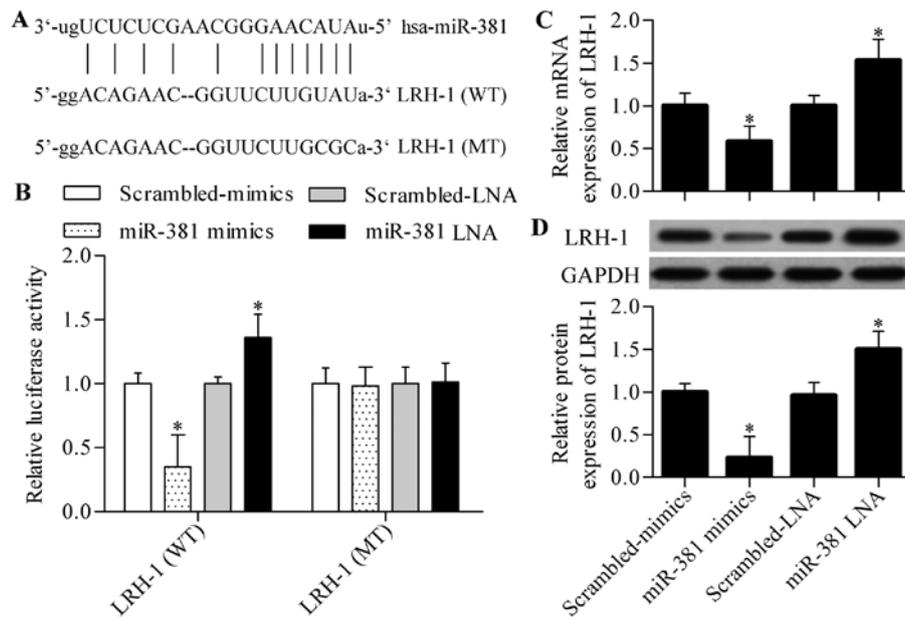


Figure 5. miR-381 targets and regulates LRH-1. (A) Diagram of the predicted miR-381 targeting site in the 3'-UTR of LRH-1. (B) Dual-luciferase reporter assay of the WT and MT LRH-1 3'-UTR reporter constructs in the presence of miR-381 mimics or miR-381 LNA. (C) qPCR analysis of LRH-1 mRNA expression levels in miR-381 mimics or miR-381 LNA transfected SMMC-7721 cells. (D) Western blot analysis detected the LRH-1 protein expression in miR-381 mimics or miR-381 LNA transfected SMMC-7721 cells. Cells were harvested for qPCR or western blot analysis after transfection for 48 h. The relative protein expression of LRH-1 was quantitated by using Image-Pro Plus 6.0 and normalized to GAPDH. * $P < 0.05$ vs. scrambled mimics or scrambled LNA.

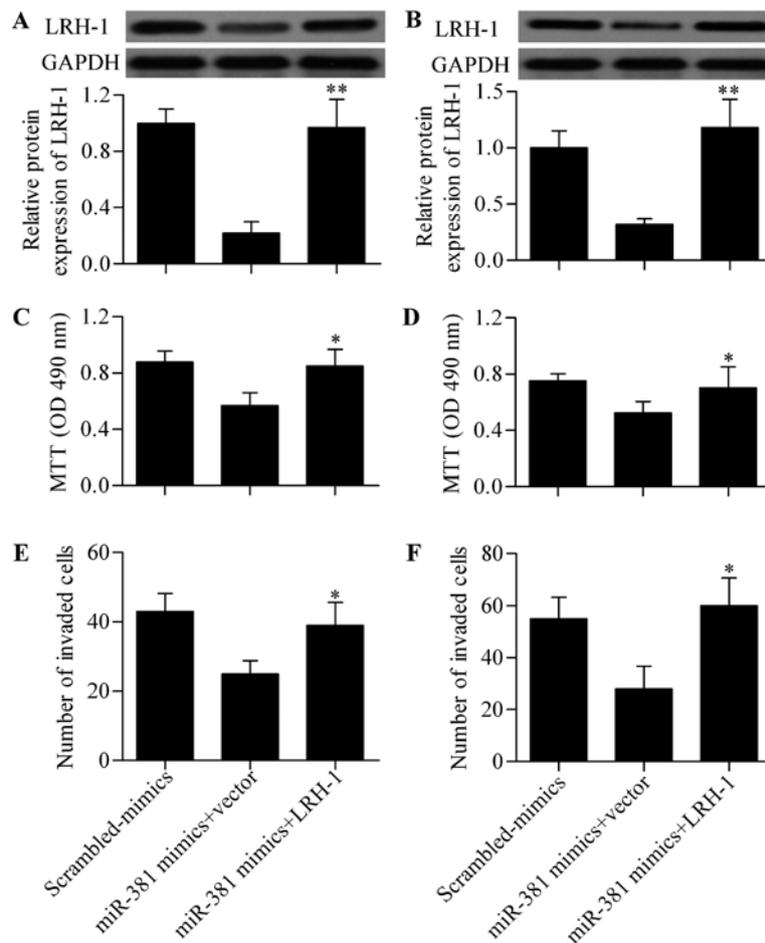


Figure 6. miR-381 inhibits HCC cell growth and invasion through LRH-1. Western blot analysis detected the LRH-1 protein expression in SMMC-7721 (A) and BEL-7402 (B) cells co-transfected with miR-381 mimics and LRH-1 expressing vectors for 48 h. The relative protein expression of LRH-1 was quantitated using Image-Pro Plus 6.0. ** $P < 0.01$ vs. miR-381 mimics + vector. MTT assay detected the effect of LRH-1 overexpression on miR-381-induced cell growth arrest in SMMC-7721 (C) and BEL-7402 (D) cells. * $P < 0.05$ vs. miR-381 mimics + vector. The effect of LRH-1 expressing vectors and miR-381 mimic co-transfection on cell invasion in SMMC-7721 (E) and BEL-7402 (F) cells. * $P < 0.05$ vs. miR-381 mimics + vector.

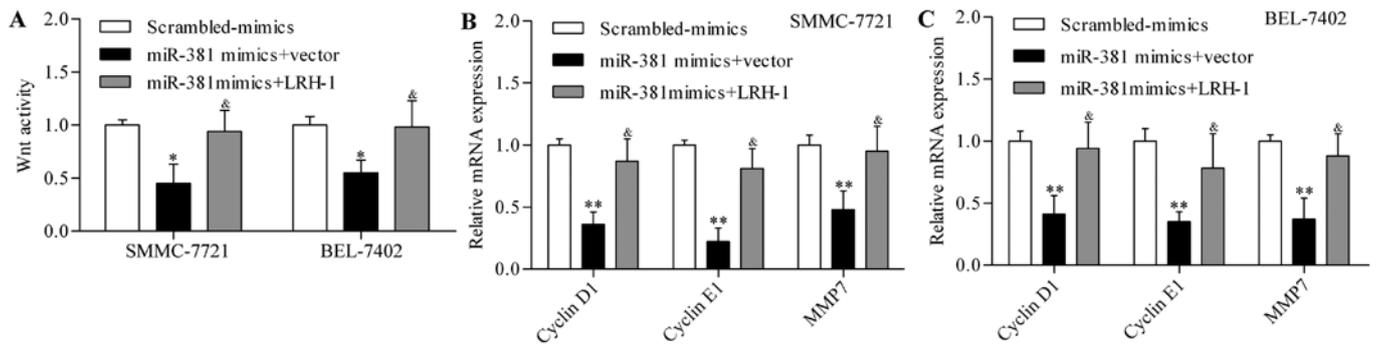


Figure 7. miR-381 negatively regulates Wnt signaling pathway through LRH-1. (A) Tcf-dependent TOPFlash reporter activity assay detected Wnt signaling activity. * $P < 0.05$ vs. scrambled mimics; [‡] $P < 0.05$ vs. miR-381 mimics + vector. qPCR analysis of cyclin D1, cyclin E1 and MMP9 mRNA expression levels in SMMC-7721 (B) and BEL-7402 (C) cells. HCC cells were co-transfected with miR-381 mimics and LRH-1 expressing vectors for 48 h. ** $P < 0.01$ vs. scrambled mimics; [‡] $P < 0.05$ vs. miR-381 mimics + vector.

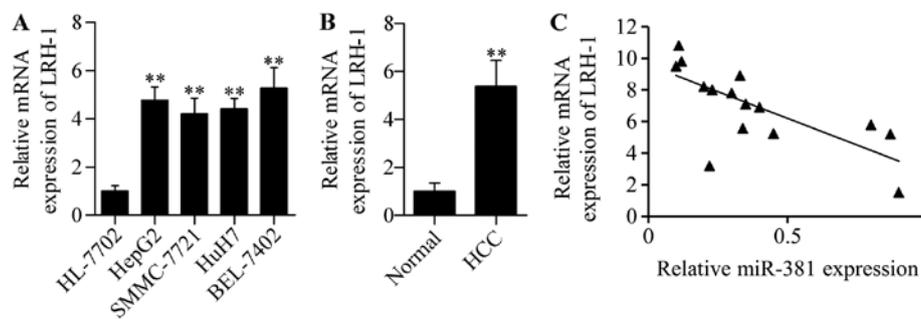


Figure 8. miR-381 inversely correlates with LRH-1. qPCR analysis of LRH-1 expression in HCC cell lines (A) and HCC tissues (B). ** $P < 0.01$ vs. HL-7702 cells or normal tissues. (C) miR-381 expression levels inversely correlated with LRH-1 expression in HCC tissues. $R = -0.6996$, $P < 0.001$.

7721 (Fig. 6C) and BEL-7402 (Fig. 6D) cells. The results further showed that the decreased cell invasion ability induced by miR-381 overexpression was also remarkably reversed by LRH-1 overexpression (Fig. 6E and F). These results indicated that miR-381 exerted its antitumor effect by inhibiting LRH-1.

miR-381 negatively regulates the Wnt signaling pathway through LRH-1 in HCC cells. To further elucidate the underlying mechanism of miR-381 in regulating HCC, we investigated the role of miR-381 in regulating the downstream signaling pathway of LRH-1. Previous studies have reported that LRH-1 can promote Wnt signalling pathways (29), which were frequently aberrantly activated in cancers including HCC (30). Wnt signaling activity was determined by using Tcf-dependent TOPFlash reporter activity assay, and the results showed that miR-381 overexpression significantly downregulated Wnt signaling activity in SMMC-7721 and BEL-7402 cells (Fig. 7A). To further verify the regulatory effect of miR-381 on Wnt signaling pathway, we next detected the transcriptional activity of the Wnt signaling pathway. The results showed that the expression of Wnt target genes, including cyclin D1, cyclin E1 and MMP9, were significantly decreased by miR-381 overexpression in SMMC-7721 (Fig. 7B) and BEL-7402 (Fig. 7C) cells. Moreover, the restoration of LRH-1 expression significantly reversed the inhibitory effect of miR-381 overexpression on Wnt signaling activity (Fig. 7). These results indicated that miR-381 regulated Wnt signaling pathway through LRH-1.

miR-381 expression level inversely correlates with LRH-1 expression in HCC tissues. To further explore the functional significance of miR-381 in HCC, we detected its association with LRH-1 in HCC tissues. We found that LRH-1 mRNA was highly expressed in HCC cells (Fig. 8A) and HCC tissues (Fig. 8B). Furthermore, correlation analysis showed that miR-381 expression was inversely associated with LRH-1 expression in HCC tissues (Fig. 8C). These results indicated that dysregulated miR-381 expression may contribute to increased LRH-1 expression and HCC development and progression.

Discussion

In the present study, we found that low miR-381 expression and high LRH-1 expression co-occurred in HCC tissues and cell lines showing an inverse correlation relationship. The study demonstrated a direct targeted relationship between miR-381 and LRH-1, in which miR-381 negatively regulated LRH-1 expression. The decreased miR-381 expression in HCC may contribute to the highly overexpressed LRH-1 and thus promoted tumor development and progression. This study provides novel insights into the molecular mechanism underlying the pathogenesis of HCC malignancy.

The role of LRH-1 has been extensively studied during the past decades. LRH-1 is required for embryonic development (33) and for maintaining the pluripotency of embryonic stem cells (34). LRH-1 is essential for pregnancy, and a lack of

LRH-1 results in infertility (19,35). Moreover, LRH-1 regulated bile acid biosynthesis and glucose sensing processes (20,22). However, the deregulated LRH-1 contributed to disease occurrences such as cancer. The most studied cancer type is breast cancer, wherein LRH-1 was overexpressed, which was associated with aromatase production (36-38). Annicotte *et al* reported that LRH-1 was an estrogen receptor target gene that exhibited potential oncogenic effects during tumorigenesis of breast cancer (39). LRH-1 was also capable of regulating the estrogen receptor expression and estrogen receptor-mediated target gene expression that promoted breast cancer cell proliferation (27,40,41). LRH-1 overexpression promoted breast cancer cell migration and invasion (42). LRH-1 can directly target the promoter region of cyclin-dependent kinase inhibitor p21 and inhibit p21 expression leading to breast cancer cell proliferation (43). Benod *et al* found that LRH-1 was overexpressed in human pancreatic cancer cells, and LRH-1 knockdown significantly repressed pancreatic cancer cell proliferation (24). Silencing of LRH-1 inhibited colon cancer cell proliferation and induced G0/G1 cycle arrest (25). LRH-1 was highly expressed in gastric cancer tissues, and LRH-1 overexpression promoted cell proliferation of gastric cancer (26). All these studies pointed out the oncogenic role of LRH-1. The present results of our study also demonstrated that LRH-1 was overexpressed in HCC tissues and cell lines, and LRH-1 downregulation by miR-381 impeded the tumorigenic potential of HCC cells, including cell growth arrest and decreased invasion ability. These results were consistent with the findings of Wang *et al*, who reported that LRH-1 knockdown inhibited cell growth and induced cell apoptosis of HCC cells (32). Interestingly, it was reported that LRH-1 could facilitate hepatitis B virus DNA replication and gene transcription, which promoted the risk for HCC tumorigenesis (44). Overall, these findings supported the notion that LRH-1 can be used as a potential and promising molecular target for HCC prevention and treatment.

Several studies have been performed to develop a novel antagonist for LRH-1 (45,46). Corzo *et al* reported that a small molecule repressor of LRH-1 and SR1848 exhibited anti-proliferation activity against LRH-1-expressing cancer cells by inactivating LRH-1 (31). miRs have received particular attention for cancer detection and treatment in recent years because of their negative regulator effect on gene expression (47). A variety of miRs have been found to be potential molecular target for HCC treatment by targeting oncogenes or tumor suppressors. For instance, Wang *et al* reported that miR-302b inhibited HCC cell invasion and metastasis by inhibiting AKT2 expression (10). miR-99b accelerated HCC cell metastasis by suppressing claudin 11 expression (48). Recent studies have demonstrated that miR-381 plays an important role in various cancer types. For example, Chen *et al* revealed that miR-381 inhibited renal cancer cell proliferation and sensitized renal cancer cells to chemotherapeutics by inhibiting WEE1 (14,49). miR-381 overexpression suppressed multidrug resistance of cancer cells (50). In lung cancers, miR-381 overexpression inhibits cancer cell migration and invasion by inhibiting the inhibitor of differentiation 1 (15). miR-381 was downregulated in prostate cancer, and the lower expression level was correlated with

higher incidence of metastatic events (51). Consistent with these studies, the present study demonstrated that miR-381 was also significantly downregulated in HCC, and miR-381 overexpression markedly suppressed HCC cell growth and invasion. The critical role of miR-381 in HCC was shown for the first time. miR-381 exerted antitumor effects by targeting LRH-1, thereby implying that LRH-1 was one of the miR-381 targets. miR-381 exhibited different functions due to regulating different targets. Tian *et al* reported that miR-381 functioned as an oncomiR that promoted glioma growth by inhibiting leucine-rich repeat C4 expression (52,53). miR-381 inhibition sensitized glioma cells to temozolomide by upregulating neurofilament light polypeptide (54). Moreover, Papp *et al* elucidated that miR-381 may promote epithelioid sarcoma by inhibiting SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily b, member 1 (55). These conflicting findings implicated that miR-381 may exert different functions by regulating different targets in different cells or conditions.

LRH-1 is a co-activator of Wnt signaling pathway that synergistically promoted the transcriptional activity of Wnt signaling pathway by inducing cyclin E1, cyclin D1 and c-myc expression (29). Wnt signaling pathway is frequently aberrantly activated in HCC, which is associated with increased cancer cell growth and metastasis (30). LRH-1 overexpression promoted gastric adenocarcinoma cell proliferation by inducing cyclin E1 (26). LRH-1 knockdown inhibited cyclin E1, cyclin D1 and c-myc expression in pancreatic cancer cells (24). In the present study, downregulating LRH-1 by miR-381 inactivated Wnt signaling and repressed the expression of target genes, including cyclin E1, cyclin D1 and MMP9. Most importantly, restoration of LRH-1 expression reversed the inhibitory effect of miR-381 on the Wnt signaling pathway, which may explain why miR-381 overexpression impeded HCC cell growth and invasion.

To date, the specific miRs that target and regulate LRH-1 have not been well recognized and investigated. A recent study elucidated that miR-451 directly targets and suppresses LRH-1 expression in osteosarcoma cells, thereby inhibiting cancer cell proliferation (56). In the present study, miR-381 targeted and regulated LRH-1 expression in HCC cells. These findings raised the possibility that inhibiting LRH-1 by specific miR to treat and prevent cancers in which LRH-1 functioned as an oncogene. The present results therefore suggest that miR-381 may be a novel tumor suppressor that blocks HCC growth and invasion by targeting LRH-1 and inhibits the LRH-1-mediated Wnt signaling pathway. The present study highlighted the functional significance of miR-381-LRH-1 in HCC, providing novel insights into the development of future therapeutic interventions for HCC.

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