miR-1-3p suppresses proliferation of hepatocellular carcinoma through targeting SOX9

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Background: Liver cancer was the fourth leading cause of cancer-related death in 2015. Hepatocellular carcinoma (HCC) is the most common type of liver cancer. miR-1-3p plays important roles in cancer, including prostate, bladder, lung cancer, and colorectal carcinoma. The function of miR-1-3p in HCC remains poorly understood.

Methods: qRT-PCR was performed to detect the miR-1-3p expression in HCC cell lines (HCCLM3, Hep3B, Bel-7404, SMMC-7721) and the normal human hepatic cell line (LO2). HCCLM3 and Bel-7404 cells were transfected with miR-1-3p mimic or scramble control followed by water-soluble tetrazolium salt (WST-1) assay. Western blot analysis was performed to determine the protein levels. TargetScan7.1 (http://www.targetscan.org/vert_71/) was used to predict the potential targets of miR-1-3p. SRY (sex determining region Y)-box 9 (SOX9), which has been previously shown to play an important role in HCC, was found to be a target of miR-1-3p. Luciferase reporter assay was used to explore the targeting of miR-1-3p on SOX9. For in vivo tumorigenesis assay, HCCLM3 cells with stable overexpression of miR-1-3p or control plasmid were injected subcutaneously into the flank of the SCID mice and animals were monitored for tumor growth.

Results: miR-1-3p was significantly downregulated in HCC cell lines (HCCLM3, Hep3B, Bel-7404, and SMMC-7721) compared to normal human hepatic cell line (LO2). Overexpression of miR-1-3p significantly inhibited the proliferation and induced apoptosis in HCCLM3 and Bel-7404 cells. SOX9 was a direct target of miR-1-3p in HCC cells. Inhibition of SOX9 significantly inhibited the proliferation of HCCLM3 and Bel-7404 cells. In vivo, overexpression of miR-1-3p decreased tumor volume in a xenograft model.

Conclusion: These results highlight the role of miR-1-3p in HCC. Overexpression of miR-1-3p inhibited the proliferation of HCC at least partly due to the regulation of SOX9. miR-1-3p may be a promising therapeutic candidate for HCC.

Keywords: miR-1-3p, hepatocellular carcinoma, SOX9, proliferation, apoptosis

Introduction

Liver cancer was the fourth leading cause of cancer-related death in 2015 after lung, colorectal, and stomach cancer. Hepatocellular carcinoma (HCC) is the most common type of liver cancer, accounting for the vast majority (75%–90%) of all liver cancers. The risk factors for HCC include hepatitis B virus (HBV)/hepatitis C virus (HCV) infection, alcohol consumption, non-alcoholic fatty liver disease (NAFLD), cirrhosis, aflatoxin-contaminated food, smoking, tyrosinemia, obesity, and diabetes. HCC burden is highest in areas with endemic HBV infection such as in sub-Saharan Africa and Eastern Asia. Mediterranean countries such as Italy, Spain, and Greece have intermediate incidence rates, while North and South America have a relatively low incidence. However, in the US, since the increasing incidences of HCV infection, obesity, and...
NAFLD, the incidence rates of HCC have increased from 1.4 per 100,000 in 1975–1977 to 4.8 per 100,000 in 2005–2007. HCC mortality increased by 35% between 2002 and 2012, and it is predicted to remain stable until 2020 in the US. In China, liver cancer is the most commonly diagnosed cancer and the leading cause of cancer death in men before the age of 60 years. Therefore, there is an urgent need to better understand the pathophysiological mechanisms of HCC and find effective treatment options.

The initiation and development of HCC are complex processes. Chromosomal instability (CIN) (both numerical and structural chromosomal changes), single nucleotide polymorphisms (SNP), somatic mutations, telomerase reactivation, and oxidative stress all contribute to hepatocarcinogenesis. MicroRNAs (miRNAs) are a class of small, highly conserved endogenous non-coding RNA of 21–25 nucleotides in length. miRNAs regulate gene expression post-transcriptionally. miRNAs generally bind to the 3’ untranslated region (3’-UTR) of their target mRNAs and repress protein production by destabilizing the mRNA and translational silencing. Studies have demonstrated the importance of miRNAs in various biological processes, such as embryonic stem cell differentiation, cell proliferation, apoptosis, autophagy, adipogenesis, angiogenesis, and hematopoiesis. Dysregulation of miRNA expression has been reported in different types of cancer, including HCC. Many studies have shown that miRNAs (eg, miR-301a-3p, miR-448, miR-506, miR-1306-3p, miR-776) regulate the proliferation, invasion, and metastasis of HCC. miR-1-3p (a liver-specific miRNA) functions as a tumor suppressor against HCC. Decreased expression of miR-122 in HCC is frequently observed and is associated with poor differentiation, larger tumor size, metastasis and invasion, and poor prognosis. The circulating miR-200 family may be used as diagnostic markers for HCC.

miR-1-3p has multiple biological functions. For example, hypertrophic cardiomyopathy (HCM) and dilated cardiomyopathy (DCM) have different miRNA expression patterns. miR-1-3p downregulated in HCM and correlated with left ventricular end diastolic diameter (LVEDD) and left ventricular ejection fraction (LVEF) that reflected the cardiac function in HCM and can serve as a potential target and differentiator HCM from DCM. miR-1-3p is downregulated by hypoxia in pulmonary artery smooth muscle cells and contributes to the development of pulmonary vascular remodeling. miR-1-3p was downregulated in various types of cancer, such as prostate cancer, oral squamous cell carcinoma (OSCC), bladder cancer, colorectal carcinoma, and lung cancer, where miR-1-3p regulated the proliferation and invasion.

In liver, miR-1-3p was upregulated at the early stage of drug-induced liver injury and served as a biomarker for hepatocellular injury. This study showed that miR-1-3p was also downregulated in HCC. HCC patients with higher miR-1-3p serum levels showed longer overall survival, and low miR-1-3p expression is associated with shortened survival time. However, the function and mechanism of miR-1-3p in HCC remains largely unknown.

In this study, we explored the role of miR-1-3p in HCC and found that SRY (sex determining region Y)-box 9 (SOX9) was a direct target of miR-1-3p. miR-1-3p inhibited the proliferation of HCC both in vitro and in vivo at least partly due to the regulation of SOX9.

Materials and methods

Cell culture

The normal human hepatic cell line LO2 and human HCC cell line SMMC-7721, HCCLM3, and Bel-7404 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Human HCC cell line Hep-3B was obtained from American Type Culture Collection (ATCC, Manassas, VA, US). Cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% fetal bovine serum (FBS), L-glutamine, and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin). All cells were maintained in a 37°C humidified incubator with 5% CO₂.

Transient transfections

miR-1-3p mimic, scramble control, and si-SOX9 (si-SOX9-1) were purchased from GenePharma (Shanghai, China). miR-1-3p inhibitor was purchased from Qiagen (Qiagen, Valencia, CA). The second si-SOX9 (si-SOX9-2) was purchased from Santa Cruz (Santa Cruz, CA). HCCLM3 and Bel-7404 cells were seeded in 6-well plates. Oligofectamine reagent (Invitrogen, Calsbad, CA) was used for cell transfection. The final concentration of miR-1-3p mimic, miR-1-3p inhibitor, scramble control, si-SOX9-1, or si-SOX9-2 is 100 nM. At the indicated time point, cells were collected.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cells using Trizol (Invitrogen). Reverse transcription was performed using the miScript RT
Kit (TaKaRa, Dalian, China). RT-qPCR was performed using miScript SYBR Green PCR Kit (Qiagen) on a C1000 thermal cycler (Bio-Rad, Hercules, CA). The primers of miR-1-3p and U6 were obtained from Qiagen (Qiagen). U6 was used as an internal control.

**Cell proliferation assay**

Cell proliferation was determined using WST-1 assay (Beyotime, Shanghai, China). After HCCLM3 and Bel-7404 cells were transfected with miR-1-3p mimic or scramble control for 6 hours, cells were seeded in 96-well plates (2,000 cells/well). At 24, 48, and 72 hours, culture medium was removed and 100 μL fresh medium containing 10 μL of WST-1 reagents was added into the wells. After 1–2 hours, the absorbance was measured at 450 nm by using an ELISA Microplate Reader (Biocompare, San Francisco, CA). For SOX9 siRNA study, HCCLM3 and Bel-7404 cells were transfected with si-SOX9-1, si-SOX9-2, or scramble control for 6 hours, and cells were seeded in 96-well plates (2,000 cells/well). At indicated time points, WST-1 assay was performed.

**Western blot analysis**

Total protein was extracted from cells using a protein extraction kit (Beyotime). Protein concentration was measured using the BCA Protein Assay Kit (Beyotime). Protein fractions were separated on sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel electrophoresis (Bio-Rad) and transferred to a nitrocellulose membrane (Bio-Rad). After blocking in 5% skimmed milk in phosphate-buffered saline (PBS) for 1 hour at room temperature, the membranes were incubated overnight at 4°C with primary antibodies. Primary antibodies against PARP and caspase-3 were obtained from Cell Signal Technology (Beverly, MA). Primary antibody against SOX9 and β-actin were obtained from Abcam (Cambridge, MA). Secondary antibodies IRDye800CW Goat anti-Mouse IgG and IRDye800CW Goat anti-Rabbit IgG were obtained from LI-COR (LI-COR Biotechnology, Lincoln, NE). Western blot images were detecting by using Li-COR Odyssey 9120 Imaging System (LI-COR Biotechnology).

**Luciferase reporter assays**

SOX9 3′-UTR was obtained from Genecopoeia (Rockville, MD). We mutated two nucleotides (1105–1106, UC to CA) of the SOX9 3′-UTR by using Site-Directed Mutagenesis kit (Stratagene, Shanghai, China). These vectors also express the Renilla luciferase serving as internal controls for the dual-luciferase assay. HCCLM3 and Bel-7404 were co-transfected with miR-1-3p mimic (100 nM) or scramble (100 nM) with SOX9 3′-UTR or its mutant using lipofectamine 2,000 transfection reagent (Invitrogen). After 48 hours of transfection, the luciferase activity was measured using the dual luciferase reporter assay kit (Promega, Madison, WI).

**Colony formation assay**

For the miR-1-3p mimic study, HCCLM3 and Bel-7404 cells were transfected with miR-1-3p mimic or scramble control for 6 hours, cells were then seeded in 10 cm dishes (1,000 cells/dish) and cultured for 10 days to allow colony formation. For stable overexpression of the miR-1-3p study, Lentiviral plasmid vector expressing miR-1-3p (LentimiRa-hsa-miR-1-3p) and scramble control lentivirus vector (LV-con) were obtained from abm (Richmond, BC, Canada). We established an HCCLM3 cell line with stable overexpression of miR-1-3p by transfecting cells with LentimiRa-hsa-miR-1-3p. The control HCCLM3 cells were transfected with LV-con. LentimiRa-hsa-miR-1-3p cells and LV-con cells were seeded in 10 cm dishes at 2,000 cells/dish and cultured for 14 days. After fixation with methanol for 20 minutes, the colonies were stained with 0.1% crystal violet.

**Mouse xenograft model**

For tumorigenesis assays, 6 weeks old, male SCID mice were purchased from Wei Tong Li Hua Experimental Animal Technology Co., Ltd (Beijing, China) (n=3); 1×10⁶ miR-1-3p stable overexpression HCCLM3 cells (LentimiRa-hsa-miR-1-3p) or control HCCLM3 cells (LV-con) were injected subcutaneously into the flank of the mice. Mice were observed for 30 days for tumor formation. The size of tumor was measured every 5 days. Tumor diameters were measured with digital calipers, and the tumor volume in mm³ was calculated by the formula: Volume=(width)²×length/2. All animal studies were approved by the Ethics Committee of Fudan University Pudong Medical Center. The handling of the mice and all experimental procedures were carried out in strict accordance with Fudan University Guidelines for the Care and Use of Laboratory Animals.

**Statistical analysis**

Data represent the mean±SD. Experiments were repeated at least three times. Statistical analysis was performed using GraphPad Prism (version 5.0, GraphPad Software, La Jolla, CA). One-way analysis of variance along with Bonferroni adjustment and Student’s Unpaired t-test were used to evaluate the differences between groups. A P-value<0.05 was considered statistically significant.
Results

miR-1-3p was downregulated in HCC cells, and overexpression of miR-1-3p inhibited the proliferation of HCC

To explore the role of miR-1-3p in HCC, we first examined the expression of miR-1-3p in human HCC cell lines (Hep3B, HCCLM3, Bel-7404, and SMMC-7721) and human immortalized hepatocyte (LO2) by qRT-PCR analysis. As shown in Figure 1A, miR-1-3p was significantly downregulated in all HCC cell lines (Hep3B, HCCLM3, Bel-7404, and SMMC-7721) compared to LO2 cells. To further investigate the potential impact of miR-1-3p on HCC, HCCLM3 and Bel-7404 were transfected with miR-1-3p mimic or scramble control. The transfection efficiency was confirmed. As shown in Figure 1B, after 72 hours of transfection, the expression of miR-1-3p was significantly increased in both HCCLM3 and Bel-7404 cells compared to those transfected with scramble control. As shown in Figure 1C, cell viability was measured using WST-1 assay. miR-1-3p overexpression significantly inhibited proliferation in both HCCLM3 and Bel-7404 cells compared with control groups transfected with scramble. As shown in Figure 1D, miR-1-3p mimic significantly inhibited the colony formation of HCCLM3 and Bel-7404 cells. Western blot analysis showed that the levels of cleaved PARP and cleaved caspase-3 were significantly increased in HCCLM3 and Bel-7404 cells transfected with miR-1-3p mimics compared with control groups (Figure 1E). These results indicated that overexpression of miR-1-3p inhibited proliferation and promoted apoptosis in HCC cells.

Figure 1 Overexpression of miR-1-3p inhibited proliferation of HCC cells. (A) The expression of miR-1-3p was downregulated in HCC cell lines (HCCLM3, Hep-3B, Bel-7404, and SMMC-7721) compared to the normal liver cell line LO2. (B) miR-1-3p expression was determined by qRT-PCR in HCCLM3 and Bel-7404 after transfection of miR-1-3p mimic or scramble control for 72 hours. (C) HCCLM3 and Bel-7404 cells were transfected with miR-1-3p mimic or scramble control. The proliferation in HCCLM3 and Bel-7404 cells was measured by WST-1 analysis. (D) Representative images of colony formation. (E) Western blot analysis was performed to determine the protein levels of PARP and caspase-3. β-actin was used as an internal control.

Notes: Data was expressed as mean±SD. *P<0.05, **P<0.01.

Abbreviations: HCC, hepatocellular carcinoma; qRT-PCR, quantitative real-time PCR.
SOX9 is a direct target of miR-1-3p in HCC cells.

To investigate the tumor suppressive mechanism of miR-1-3p, we next attempted to identify the potential target genes directly regulated by miR-1-3p by using miRNA target prediction programs TargetScan (http://www.targetscan.org/vert_72/). We found that there was a predicted miR-1-3p binding site in the 3′-UTR of SOX9 (Figure 2A). To determine whether SOX9 was regulated by miR-1-3p, HCCLM3 and Bel-7404 were transfected with scramble control, miR-1-3p mimic, or miR-1-3p inhibitor. Western blot analysis showed that overexpression of miR-1-3p significantly downregulated SOX9 protein levels in both HCCLM3 and Bel-7404 cells, while inhibition of miR-1-3p significantly increased the expression of SOX9 in both of HCCLM3 and Bel-7404 cells (Figure 2B). To further verify whether SOX9 was a direct target of miR-1-3p, we generated SOX9 reporter construct containing 3′-UTR with mutations of miR-1-3p binding site (indicated in Figure 2A). HCCLM3 and Bel-7404 cells were transfected with wild type (WT) or mutant (Mut) SOX9 3′-UTR and miR-1-3p mimic or scramble control. Luciferase reporter assay showed that miR-1-3p mimic remarkably decreased the 3′-UTR luciferase reporter activity of SOX9, this effect was abolished when the miR-1-3p binding site was mutated (Figure 2C). These findings suggested that SOX9 was a direct target of miR-1-3p in HCC cells.

SOX9 was upregulated in HCC patients and correlated with poor prognosis and inhibition of SOX9 suppressed HCC cells proliferation

In humans, SOX9 located on chromosome 17q24. SOX9 is a transcript factor, belongs to a highly conserved family of transcription factors defined by their similarity to the high mobility group DNA-binding domain of sex determining region Y (SRY).\textsuperscript{34} SOX9 is expressed during embryogenesis and plays important roles during the development of many organs including testis, pancreas, heart, liver, cartilage, ovary, prostate, skin, and breast.\textsuperscript{34} Mutations in SOX9 cause campomelic dysplasia (CD).\textsuperscript{35} Based on The Cancer Genome

Figure 2 SOX9 was a direct target of miR-1-3p in HCC. (A) miR-1-3p and its putative binding site in the 3′-UTR of SOX9. Mutations were generated on the two nucleotides of the SOX9 3′-UTR. (B) Western blot analysis of SOX9 expression in HCCLM3 and Bel-7404 cells after transfection with miR-1-3p mimic, miR-1-3p inhibitor, or scramble control for 48 hours. (C) Luciferase activity assay. HCCLM3 and Bel-7404 cells were co-transfected with a WT or Mut SOX9 3′-UTR and miR-1-3p mimic or scramble control.

Notes: Data was expressed as mean±SD. *P<0.05.

Abbreviations: HCC, hepatocellular carcinoma; 3′-UTR, 3′ untranslated region; WT, wild type; Mut, mutant.

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Atlas (TCGA) database, from http://ualcan.path.uab.edu/, we found that SOX9 increased in human primary HCC tumors compared with normal control (Figure 3A). The expression of SOX9 increased with increasing grade of HCC (Figure 3B). HCC patients with lower or medium expression of SOX9 had longer overall survival time than patients with higher SOX9 expression (Figure 3C). These results demonstrated that SOX9 is an oncogene in HCC. To confirm the role of SOX9 in HCC, HCCLM3 and Bel-7404 cells were transfected with si-SOX9 (si-SOX9-1 and si-SOX9-2) or nonsense control (NC). Forty-eight hours post-transfection, protein samples were collected. Western blot analysis revealed that the protein level of SOX9 was significantly decreased in response to si-SOX9 treatment (Figure 3D). WST-1 assay showed that inhibition of SOX9 significantly decreased the proliferation in both HCCLM3 and Bel-7404 cells (Figure 3E). These results confirmed that SOX9 promotes tumorigenesis of HCC.

Overexpression of miR-1-3p suppresses HCC growth in vivo
To evaluate the effects of miR-1-3p on HCC growth in vivo, we generated HCCLM3 cells with stable overexpression of miR-1-3p. HCCLM3 cells were transfected with LentimiRa-hsa-miR-1-3p or LV-con. The upregulation of miR-1-3p was confirmed by qRT-PCR (Figure 4A). As shown in Figure 4B, overexpression of miR-1-3p led to a significantly decreased colony formation in HCCLM3 cells compared with the control group. HCCLM3 cells with stable overexpression of miR-1-3p (LentimiRa-hsa-miR-1-3p) or control plasmid (LV-con) were injected subcutaneously into the flank of SCID mice to establish a xenograft model. Compared with the control group, overexpression of miR-1-3p resulted in a significant reduction of tumor size, tumor volume (Figure 4C), and tumor growth (Figure 4D). Western blot analysis of the tumor tissues confirmed the significant downregulation of SOX9 in miR-1-3p overexpression tumors compared with the control group (Figure 4E). Taken together, these results suggested that overexpression of miR-1-3p played an important role in suppressing HCC proliferation.

Discussion
Valery et al. predicted the future burden of primary liver cancer in 30 countries around 2030. An increase in the number of new cases of primary liver cancer each year is
predicted to 2030 in most studied countries, as a result of both changes in risk factors as well as population aging and growth. With increasing control of HBV and HCV, viral hepatitis-related primary liver cancer rates may decrease in the future; however, the increasing levels of obesity and its metabolic complications and increasing alcohol consumption may cause an increased incidence of HCC. HCC is a complex and heterogeneous tumor with several genomic alterations. Aberrant activation of several signaling cascades, such as EGFR-Ras-MAPKK, PI3K/Akt/mTOR, Wnt-β-catenin, Hedgehog signalling, and apoptotic pathways, have been reported in HCC. Deregulation of the networks of tissue-specific transcription factors (eg, HNF4α, C/EBPs, p73, and TCF7L2) observed in HCC leads to profound changes in the hepatic transcriptional program that facilitates tumor progression. Identifying the important molecules involved in HCC development is of great importance for developing effective therapeutic approaches for HCC.

miRNAs play important roles in HCC. miRNA are mostly downregulated in cancer. It has been reported that ERK activation globally downregulates miRNAs through phosphorylating exportin-5 (XPO5). Pin1 impairs microRNA biogenesis by mediating conformation change of XPO5 and promoted HCC development. In this study, we found that miR-1-3p was significantly downregulated in HCC cell lines. Overexpression of miR-1-3p suppressed proliferation and induced apoptosis in HCC cells. SOX9 is a direct target of miR-1-3p in HCC cells. In a mouse model, overexpression of miR-1-3p significantly suppressed tumorigenicity. miR-1-3p inhibited the proliferation of HCC at least partly through targeting SOX9. SOX9 plays important roles in multiple aspects of embryonic development. During embryogenesis, SOX9 is

Figure 4 Overexpression of miR-1-3p reduced tumor burden in vivo. (A) miR-1-3p level was determined by qRT-PCR in HCCLM3 cells with stable expression of miR-1-3p (LentiRa-hsa-miR-1-3p) and control (LV-con). (B) Representative images of colony formation. (C) Representative image of tumors excised from xenograft LentiRa-hsa-miR-1-3p group and LV-con group (upper panel). Volume of xenograft tumor (lower panel). (D) Tumor growth curve. The size of tumor was measured every 5 days. (E) Western blot analysis was performed to determine the protein level of SOX9 in LentiRa-hsa-miR-1-3p and control xenograft tumor tissues.

Notes: Data was expressed as mean±SD. *P<0.05, **P<0.01.

Abbreviation: qRT-PCR, quantitative real-time PCR.
expressed in a broad array of tissues including the gonad, otic vesicle, lung, notochord, neural tube, pancreas and cardiac cushions. SOX9 is expressed in a wide range of cancers, including pancreatic cancer, breast cancer, prostate cancer, lung adenocarcinoma, esophageal squamous cell carcinoma, and melanoma. In liver, SOX9 regulates biliary development and contributes to liver regeneration and fibrosis. The prevalence of SOX9 in biopsies from patients with chronic liver disease correlated with fibrosis severity and accurately predicted disease progression toward cirrhosis.

In HCC, SOX9 plays a critical role in self-renewal and tumorigenicity by promoting symmetric cell division of cancer stem cells. Based on the TCGA database, SOX9 expression increased with increasing grade of HCC tumors. A high level of SOX9 was significantly associated with poor HCC patient survival. Studies have shown that miRNAs can target SOX9 and modulate the development of cancer, such as miR-216b inhibits the cellular proliferation and invasion of non-small cell lung cancer (NSCLC) through the direct targeting of SOX9. miR-145 reduces proliferation, adhesion, and invasion of glioblastoma cells by suppressing the activity of oncogenic proteins Sox9 and ADD3.

In this study, we reported that SOX9 was a direct target of miR-1-3p in HCC cells. Overexpression of miR-1-3p inhibited proliferation in HCC through regulating SOX9.

Taken together, these results highlight the role of miRNAs in HCC. Decreasing of SOX9 by a miR-1-3p based approach may be a promising therapeutic target for the treatment of HCC.

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Disclosure
The authors report no conflicts of interest in this work.

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