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Original Article

miR-125b suppresses the proliferation of hepatocellular carcinoma cells by targeting Sirtuin7

Lixin Zhao, Weixing Wang

Department of Hepatobiliary and Laparoscopic Surgery, Renmin Hospital of Wuhan University, Wuhan 430060, Hubei, China

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Abstract: Previous studies have shown that microRNAs are involved in many human cancers. However, the role of miR-125b in hepatocellular carcinoma (HCC) has not been fully understood. In our study, we detected the expression of miR-125b using Real-time quantitative-polymerase chain reaction (RT-PCR) and found that the expressions of miR-125b were significantly inhibited in HCC tissues and cell lines. The levels of miR-125b were associated with the degree of HCC malignancy. Otherwise, MTT assay showed that the proliferation was significantly decreased after transfection of miR-125b mimics into HepG2 cells; while the proliferation was significantly increased in HepG2 cells transfected with miR-125b inhibitors. Furthermore, TargetScan was conducted to predict the target gene of miR-125b and Sirtuin7 (SIRT7) was chosen to a potential target gene. And then we used luciferase reporter assay and western blot to confirm that SIRT7 is a direct target gene. Western blot indicated that transfection of miR-125b mimics could significantly inhibit the expression of SIRT7 in HepG2 cells, whereas, transfection of miR-125b inhibitor could significantly increase the expression of SIRT7 in HepG2 cells. These results suggest that miR-125b can inhibit the proliferation of HCC by adjusting the expression of SIRT7 and may be a key element of HCC progression.

Keywords: Hepatocellular carcinoma cells, microRNAs, proliferation, Sirtuin7

Introduction

The morbidity and mortality of hepatocellular carcinoma (HCC) rank the sixth and third places of malignant cancer respectively [1-5]. According to the statistics, there are approximately 750,000 new cases of HCC and 700,000 patients died in china every year. The major risk factors of HCC are viral hepatitis infections (hepatitis B and C) and liver cirrhosis which most commonly caused by excessive alcohol consumption [6]. And HCC is characterized by its difficulty diagnosis at a early stage, high incidence of cancer metastasis, recurrence after hepatic cancer surgery and resistance to chemo- and radiation therapy [7, 8]. Despite substantial progress in treatment and diagnostic technology, there are poor prognoses for the majority of patients with HCC. The main reason for the poor prognosis of patients with HCC is tumor local invasion and metastasis [9]. And 80% of patients with HCC are inoperable for various reasons clinically. Therefore, it is the most urgent need to find a new method for the treatment of HCC.

A microRNA (miRNA) is a endogenously noncoding single-stranded RNA, negatively regulating gene expression. More and more evidences suggest that aberrant expression of miRNA is closely related to the occurrence and development of many human cancers. There are several reports that the expressions of miR-125b are abnormal in a variety of tumor tissues, inhibiting tumor cell proliferation and invasion [10-12]. However, it is rarely reported that the levels of miR-125b expression in the HCC tissues and cell lines and the effect of miR-125b on HCC cell proliferation.

In the present study, we try to investigate the potential role of miR-125b by detecting the expression of miR-125b in HCC tissues and cells. Furthermore, we determined the function of miR-125b on HCC cell proliferation by using gain- and loss-of-function approaches and found that overexpression of miR-125b increased cell proliferation. In addition, we explored the mechanisms of cell proliferation for miR-125b and found that SIRT7 (a member of the sirtuin family of proteins) was a target

protein of miR-125b. These findings established the theoretical basis for the development of new drugs to treat the patients with HCC by increasing the expression of miR-125b.

Materials and methods

Patients and samples

HCC tissues and adjacent noncancerous tissues were obtained form 40 patients who had undergone surgical treatment at our hospital between December 2011 and December 2012. And normal liver tissues were obtained from 40 patients with liver hemangioma who hospitalized in the same period. All patients between the ages of 45 and 69 (mean age, 56.5 years) have not received chemotherapy and radiotherapy before surgeries. The tissues were preserved in liquid nitrogen. All experiments were approved by the Ethical Committee of Renmin Hospital of Wuhan University. Informed consent was obtained from all individual participants included in the study.

Cell lines

All cell lines (HL-7702, HepG2, SMMC-7721 and MHCC97H) were purchased from Shanghai Fengshou Bio-Technique Co. Ltd (Shanghai, China). The three cell lines (HL-7720, HepG2 and SMMC-7721) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), while MHCC97H cell line was cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% FBS. All cultured media used in the study were supplemented with penicillin and streptomycin and obtained from Hyclone (Logan, UT, USA). All cells were maintained in a humidified incubator with 5% CO_2 at 37°C.

RT-PCR

Total RNA was extracted from HCC tissues, adjacent noncancerous tissues, normal liver tissues and cell lines using TRIZOL (Invitrogen; Carlsbad, CA, USA). The relative expression levels of miR-125b were detected using Cells-to-CT™ 1-Step Power SYBR® Green Kit (Ambion; Austin, TX, USA) according to the manufacturer's instructions, which is conducted by the ABI 7500 Real-Time PCR system (Applied Biosystems by Life Technologies, Foster City, CA, USA). The gene-specific primers were designed and synthesized: Forward, 5'-GCAAUUUGGCGUCCUCCACUAA-3' and Reverse, 5'-AGUGGAGGACGCCAAAUUCCCU-3' for miR-125b; Forward, 5'-CCATGTTCGTCATGGGTG TGAACCA-3' and Reverse 5'-GCCAGTAGAGGCAGGGATGATGTTC-3' for GAPDH. GAPDH primers were used as an internal control. PCR amplification consisted of a heating step at 95°C for 5 min, followed by 40 cycles of denaturation 95°C for 10 sec and 60°C for 20 sec and 72°C for 1 min for primer extension. The $2^{-\Delta\Delta CT}$ method was used to quantify the level of miR-125b expression.

Transfection by electroporation

Electroporation was performed using Amaxa® Nucleofector® Technology consisting of cell line nucleofector kit V and Nucleofector® I Device (Lonza; Cologne, Germany) according to the manufacturer's instructions. Briefly, the growed cells were trypsinized to single cell suspension, washed twice with PBS and then resuspended in cell line nucleofector solution V at final densities of 2×10^6 cells/100 ul nucleofection. For each transfenction, 300 pmol microRNAs were added to the cell suspension solution at room temperature. The cell/miRNAs mixture (100 ul) was transferred to an Amaxa certified cuvette, which was introduced to the Nucleofector® I Device. The nucleofection was performed using D-032 program. 500 μL of pre-warmed RPMI-1640 media with 10% FBS were added to the cuvette immediately after transfection. And then the cells were transferred to a prepared 6-well plates. After incubation of 6 h in a humidified 37°C/5% CO_2 incubator, medium in each well was replaced with 2 ml basic meidum (RPMI-1640 with 10% FBS) and the cells were cultured until analysis.

MTT assay

The HepG2 cells transfected with different miRNAs at an appropriate density (2000 cells/well) were plated into 96-well plates and cultured for 1 day, 2 days, 3 days, 4 days and 5 days, respectively. At each cultured time point, 20 μl of MTT reagent was added into each well and cells were incubated at 37°C in the dark for 4 h. Next, supernatant was removed and 150 μl DMSO was added to each well to dissolve MTT crystals. The absorbance was then measured at 490 nm with a microplate reader. Triplicate independent experiments for each sample were conducted in quintuplicate for different time point.

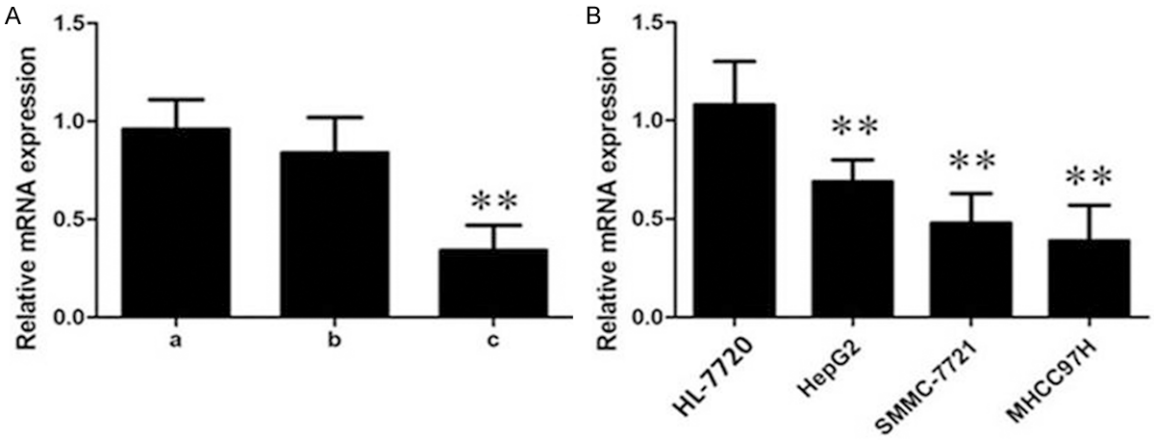

Figure 1. The expressions of miR-125b is inhibited in HCC tissues and cell lines. RT-PCR were performed to determine the levels of miR-125b expression. A: The expression of miR-125b was significantly down-regulated in HCC tissues compared with that of the adjacent noncancerous tissues and normal liver tissues (n=40). a, normal liver tissues; b, adjacent noncancerous tissues, c, HCC tissues. B: mRNA levels of miR-125b were significantly lower in three HCC cell lines (HepG2, SMMC-7721, MHCC97H) than that of the normal cell line (HL-7702). $^{**}P<0.01$.

Bioinformatics predictions and Cloning of SIRT7 3'-UTR

The target gene prediction for miR-125b was performed using TargetScan [13, 14], which revealed that the 3'-UTR of SIRT7 can be complementarily base paired with miR-125b. Total RNAs of normal liver tissues were extracted and then reverse-transcribed into cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific; Waltham, MA, USA). PCR was used to amplify the 3'-UTR of SIRT7 which was sequenced and inserted into the pMIR-REPORT™ Luciferase miRNA Expression Reporter Vector (pMIR, Life Technologies, Carlsbad, CA, USA). The constructed recombinant plasmid was named pMIR-Sir7. In addition, the mutant 3'-UTR of SIRT7 (CUCAGGG to CAGUCGG) was collected using GeneArt® Site-Directed Mutagenesis System (Invitrogen, Life Techonologies, Carlsbad, CA, USA) and then cloned into the pMIR-REPORT™ Luciferase miRNA Expression Reporter Vector. The constructed mutant plasmid was named pMIR-Sir7-M.

Luciferase reporter assay

HepG2 cells were seeded in a 6-well plate with regular growth medium without antibiotics for 16 hours prior to transfection. And then the reporter plasmids, pMIR-SIRT7 3'-UTR-wt, or pMIR-SIRT7 3'-UTR-mut with miR-125b mimics or NC oligos cotransfected into the cells for 24 h using Lipofectamine 2000, respecrively (Invitrogen, LifeTechnologies, Carlsbad, CA, USA). Then, the luciferace activities of cell lysates were measured 48 h after transfection by Dual-Luciferase Reporter System (Promega; Madison, WI, USA). Firefly luciferase activities were normalized to Renilla luciferase activities. Triplicate experiments were performed and the results are presented as the ratio of luciferase activity of miR-125b transfected cells to NC transfected cells.

Western blot analysis

HepG2 cell lines were co-transfected with pMIR-SIRT7 3'-UTR-wt or pMIR-SIRT7 3'-UTR-mut with miR-125b mimics or NC oligos as described above. And then the cells were lysed to obtain proteins with CytoBuster™ Protein Extraction Reagent (Merck KgaA; Darmstadt, Germany). The protein solutions were equated to the same concentration which was determined using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific; Waltham, MA, USA) according to the manufacture's instructions and then heated at 100°C for 10 min. The proteins were separated on a 8% or 10% SDS-PAGE and transferred to the NC membranes (Millipore; Boston, MA, USA). The anti-SIRT7 antibody was ordered from Cell signaling technology (Danfoss; MA, USA) and anti-β-actin, HRP-conjugated goat anti-rabbit IgG antibody were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). The membranes were blocked

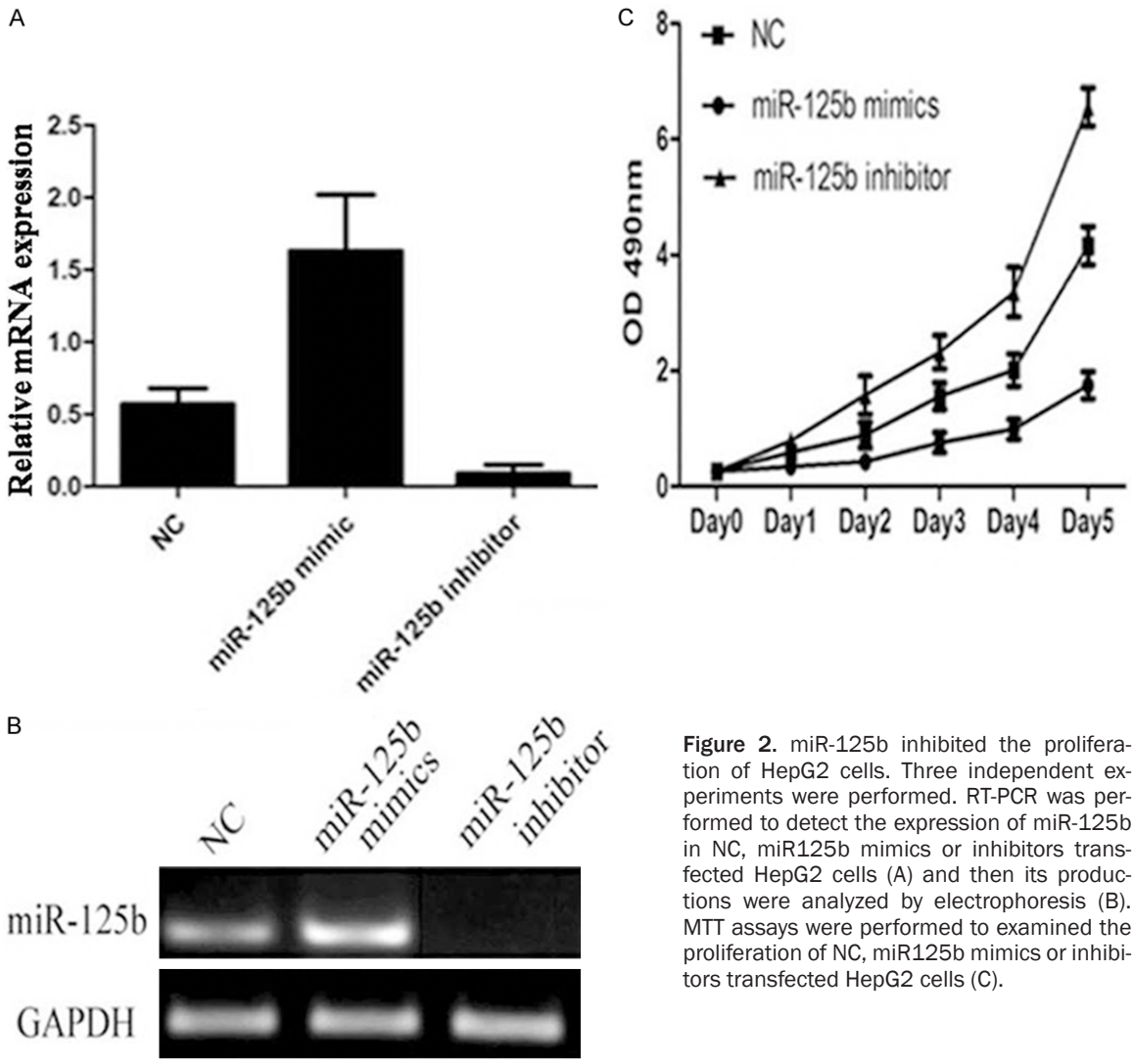

Figure 2. miR-125b inhibited the proliferation of HepG2 cells. Three independent experiments were performed. RT-PCR was performed to detect the expression of miR-125b in NC, miR125b mimics or inhibitors transfected HepG2 cells (A) and then its productions were analyzed by electrophoresis (B). MTT assays were performed to examined the proliferation of NC, miR125b mimics or inhibitors transfected HepG2 cells (C).

with 5% BSA for 2 h at room temperature and then incubated with anti-SIRT7 antibodies or anti-β-Actin overnight at 4°C. Following washing three times with TBST, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG antibody for 1 h at room temperature. The membranes were washed three times and then visualized with SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher Scientific; Waltham, MA, USA).

Statistical analysis

All of the statistical data were analyzed using SPSS15.0. Student's t-test was used to compare the data between two groups and one-way analysis of variance was performed to analyze the data among multiple groups. Two sided *P* values less than 0.05 were deemed to be significant.

Results

The expressions of miR-125b decreased in HCC tissues and cell lines

To study the levels of miR-125b expressions, total RNAs were extracted from HCC tissues, adjacent noncancerous tissues, normal liver tissues, HCC cell lines (HepG2, SMMC-7721, MHCC97H) and one normal control cell line (HL-7702). The results showed that the expression of miR-125b was significantly lower in the HCC tissues than that of the adjacent noncancerous tissues and normal liver tissues (**P<0.01, **Figure 1A**). Otherwise, the data from cell lines indicated that miR-125b expression remarkably decreased in three HCC cell lines compared with HL-7702 cell line (**P<0.01, **Figure 1B**). And a stepwise decrease trend in miR-125b expression was observed along with

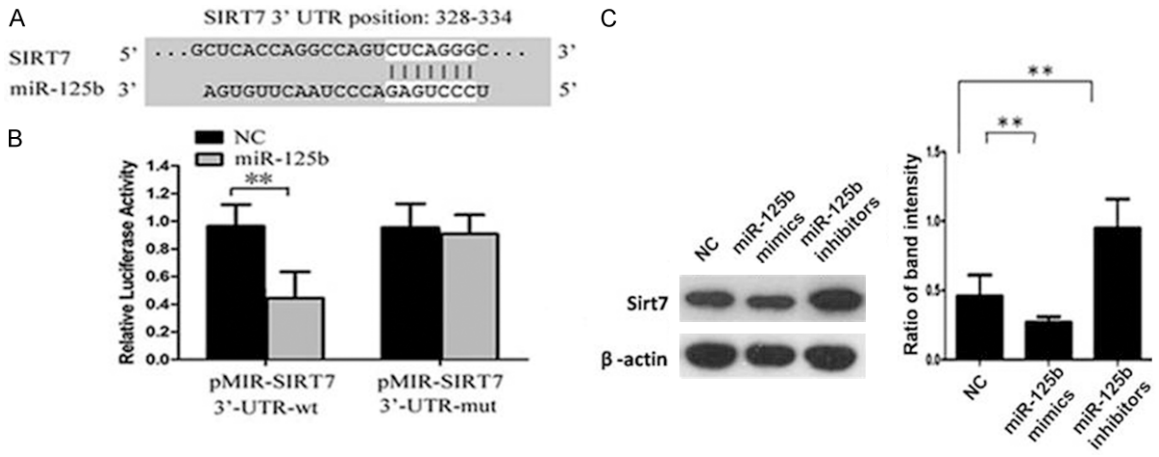

Figure 3. SIRT7 is a direct target of miR-125b. A: The 3'UTR of SIRT7 (nt328-334) contains a putative binding site of miR-125b. B: HepG2 cells were cotransfected with the reporter plasmids, pMIR-SIRT7 3'-UTR-wt, or pMIR-SIRT7 3'-UTR-mut and miR-125b mimics or NC oligos for 24 h. Relative luciferase activities were detected using Dual-Luciferase Reporter System. The luciferase activities were significantly suppressed by miR-125b in the pMIR-SIRT7 3'-UTR-wt transfected HepG2 cells, but not in the pMIR-SIRT7 3'-UTR-mut transfected cells. C: Western blot analysis was performed to detect the expression of SIRT7 in HepG2 cells transfected with miR-125b mimics, miR-125b inhibitors or NC oligos. Overexpression of miR-125b downregulate the expression of SIRT7, which was upregulated in HepG2 cells transfected with miR-125b inhibitors compared with that of HepG2 cells transfected NC oligos. $^{**}P<0.01$.

increased HCC cell lines' malignancy degree, implicating that miR-125b may be participated in the progression of HCC.

MiR-125b expression inhibited the proliferation in HepG2 cells

To investigate the effect of miR-125b on the proliferation in HepG2 cells, we transfected miR-125b mimics and miR-125b inhibitors into HepG2 cells respectively and determined the expression of miR-125b by RT-PCR. mRNA levels of miR-125b significantly increased in HepG2 cells transfected with miR-125b mimics and significantly decreased in HepG2 cells transfected with miR-125b inhibitors (**Figure 2A**, **2B**). After transfection, MTT assays were performed to examine the proliferation rate in either NC normal cells or transfected cells (**Figure 2C**). The results indicated that overexpression of miR-125b reduced the proliferation of HepG2 cells; while inhibition of miR-125b promoted the proliferation of HepG2 cells.

SIRT7 is a directly target of miR-125b

miRNAs play an important role in RNA silencing and post-transcriptional regulation of gene expression by complementary base-paring with their targeted mRNA molecules [15-17]. Therefore, a target prediction method, TargetScan (http://www.targetscan.org/), was conducted to predict the potentially target genes of miR-125b. We found that 3'-UTR of SIRT7 contained the miR-125b target sites (CUCAGGG, nt 328-334, **Figure 3A**). To confirm whether SIRT7 was directly targeted by miR-125b, we constructed the wild type and mutative 3'-UTR of SIRT7 and then performed luciferase reporter assays as described above in HepG2 cells. As shown in **Figure 3B**, the density of luciferase expression significantly reduced in HepG2 cells cotransfected with pMIR-SIRT7 3'-UTR-wt and miR-125b mimics compared with that of HepG2 cells cotransfected with pMIR-SIRT7 3'-UTR-wt and NC oligos ($^{**}P<0.01$). Moreover, miR-125b did not significantly alleviate the luciferase activity in HepG2 cells transfected with pMIR-SIRT7 3'-UTR-mut compared with that in NC oligos transfected HepG2 cells. Otherwise, the western blot results showed that upregulation of miR-125b inhibited the expression of SIRT7.In contrast, downregulation of miR-125b increased the expression of SIRT7 (**Figure 3C**).

Discussion

HCC is one of most common cancer in the world, ranking the third in the most common causes of cancer death [18]. 78% of liver cancer is resulted from hepatitis virus infection [19]. In recent years, the patients with HCC in early stage could be cured by the treatment of

surgical excision, vascular intervention or liver transplant. However, only 30% of these patients with HCC associated with cirrhosis are suitable for being treated by these methods [20]. Therefore, new effective treatments are desperately needed to be found due to the lack of effective treatment method for HCC.

MicroRNA (miRNA) is an endogenously small non-coding RNA and involoved in many physiological and pathological processes. Increasing evidences suggest that the significantly abnormal expressions of microRNAs were observed in different tumor tissues, implicating that miRNAs are closely related to the occurrence and development of tumors [21-23]. Bai et al [24] reported that the expression level of miR-409-3p significantly decreased in colorectal cancer tissues and miR-409-3p inhibited colorectal cancer invasion and metastasis by targeting GRB2-associated binder protein 1 (GAB1). Wang et al [25] found that the expression of miR-218 in esophageal squamous cell carcinoma was significantly reduced, presenting a trend that the higher tumor grade, the lower the expression level of miR-218. miR-218 plays a role of cancer suppressor gene in the esophageal squamous cell carcinoma by regulating B cell-specific Moloney murine leukemia virus integration site 1 (BMI1).

Previous data have shown that miR-125b had the abnormal expressions in a variety of tumor tissues and played an important role as an tumor suppressor gene [10-12]. However, the expression level of miR-125b in HCC, its functions and mechanism behind this are not fully understood. In our study, we examined the expression of miR-125b in HCC tissues, adjacent noncancerous tissues and normal liver tissues by RT-PCR and found that a significant reduction of miR-125b was observed in HCC tissues and cell lines compared with that of adjacent noncancerous tissues, normal liver tissue and cell lines, indicating that miR-125b may be an tumor suppressor gene. And then we demenstrated that miR-125b inhibited the proliferation of HCC cells using gain- and loss-of-function approach. It is known that miRNAs play a critical biological role by regulating their target genes, which subsequently give rise to a series of pathophysiology changes. Accordingly, our data proved that SIRT7 is a directly target gene of miR-125b by the prediction method, TargetScan, and luciferase assay. It is reported that SIRT7 plays a vital role in the development of human cancer [26-28]. So miR-125b functions through regulating the expression of SIRT7, which subsequetly suppress the proliferation of HCC.

In conclusion, our investigation confirmed that miR-125b was a tumor suppressor gene in HCC by regulating its target gene, SIRT7. miR-125b/SIRT7 pathway will provide a novel method for the treatment of HCC.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Weixing Wang, Department of Hepatobiliary and Laparoscopic Surgery, Renmin Hospital of Wuhan University, No. 99 Zhangzhidong Road, Wuchang District, Wuhan 430060, Hubei, P. R. China. Tel: +86-02788041911-86643; Fax: +86-02788042292; E-mail: weixingw288@163.com

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