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*Original Article*

# miR-138 suppresses cell proliferation and invasion by inhibiting SOX9 in hepatocellular carcinoma

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**Abstract:** Accumulating evidence suggests that miR-138 expression was frequently downregulated in different cancer types and involves in the progression of tumorigenesis. However, the biological role and molecular mechanism of miR-138 involvement in hepatocellular carcinoma (HCC) still remains largely unknown. Therefore, in the present study, we investigated the role of miR-138 in the progression of HCC. We found that miR-138 expression levels were significantly downregulated in HCC tissues and cell lines compared with the corresponding noncancerous liver tissues and normal hepatic cell line. In addition, we also found that enforced expression of miR-138 inhibited proliferation, colony formation, migration and invasion in HCC cells. Using a luciferase reporter assay, *SOX9* was confirmed as a direct target of miR-138. Quantitative real-time polymerase chain reaction (qRT-PCR) and western blot assay showed that overexpression of miR-138 in HCC cells significantly inhibited SOX9 expression on mRNA level and protein level. Furthermore, SOX9 expression was significantly upregulated in HCC tissues and cell lines, and its mRNA expression is negative correlated with miR-138 expression in clinical HCC tissues ($r$=-0.689, $P$<0.01). Of note, downregulation of SOX9 performed similar effects with overexpression of miR-138. These findings suggested that miR-138 functioned as a tumor suppressor in HCC partially via repressing SOX9 expression.

**Keywords:** Hepatocellular carcinoma, miR-138, SOX9, proliferation, invasion

## Introduction

Hepatocellular carcinoma (HCC) is one of the most lethal malignancies, which has been ranked as the fifth most common malignancy and the third leading cause of cancer-related death worldwide [1]. Despite improvement have been made in diagnosis and multimodality treatment in the past decades, the prognosis of HCC patients remains gloomy mainly due to its high recurrent [2] and metastatic rate [3]. Therefore, there is an urgent need to understand the molecular mechanism by which regulate the growth and metastasis of HCC for exploring the novel diagnosis molecular and therapy agent.

MicroRNAs (miRNAs) are a class of endogenous small non-coding RNA molecules (19-25 nucleotides in length) that regulate gene expression post-transcriptionally by binding to the 3'-untranslated region (3'UTR) of target mRNAs leading to translational repression or degradation of mRNA [4]. Accumulating evidence shows that miRNAs play crucial roles in the coordination of a wide variety of biological processes, such as differentiation, morphogenesis and tumorigenesis [5, 6]. It was well known that miRNAs involved in regulation of cancer initiation and progression, and functioned as oncogene or tumor suppressor roles by repressing target gene [7, 8]. To date, numerous miRNAs has been found to played an important role in the carcinogenesis of HCC [9, 10], suggesting that miRNAs could serve as potential novel targets for HCC therapy.

miR-138, a family of microRNA precursors, attracts much attention and has been extensively studied. miR-138, has been reported to be downregulated and function as a tumor suppressor in a variety of human cancers, such as larynx carcinoma [11], colorectal cancer [12], non-small lung cancer [13], oral squamous cell carcinoma [14], gallbladder carcinoma [15], renal carcinoma [16], esophageal squamous cell carcinoma [17]. Although recently a study has been demonstrated that miR-138 expression

was downregulated, and could induce cell cycle arrest by targeting cyclin D3 in hepatocellular carcinoma [18], the detail biological function, especially in regards to affect migration and invasion, and underlying molecular mechanism of miR-138 in HCC has not been total elucidated. Therefore, here the role of miR138 on the carcinogenesis, especially with respect to affect migration and invasion of HCC, and the underlying mechanisms were investigated.

## Materials and methods

### *Patients and tissue samples*

A total of 40 surgical specimens of HCC tissues and their paired adjacent non-cancerous tissues were obtained from patients with HCC who underwent surgery between July 2010 and September 2014 at Department of Hepatobiliary and Pancreatic Surgery, the First Hospital, Jilin University (Changchun, china). Fresh specimens were immediately frozen in liquid nitrogen after resection, and stored at -80°C until use. All specimens had been histologically and clinically diagnosed by two independently experienced pathologists. Informed consent was obtained from all patients, and the project protocols were approved by the ethics committee of Jilin University.

### *Cell lines and cell cultures*

HCC cell lines (SMMC-7721, Hep3B, HepG2, Huh-7) and normal hepatic cell line, HL-7702 were obtained from Institute of Cell Biology of Chinese Academy of Science (Shanghai, China). All cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, HyClone, USA), 100 U/ml penicillin or 100 μg/ml streptomycin, and incubated at 37°C in a humidified incubator with an atmosphere of 5% $CO_2$.

### *Cell transfection*

The miR-138 mimic (miR-138) and corresponding negative control (miR-NC), small interfering RNA against SOX9 (si-SOX9) and its negative control (si-NC) were purchased from Genechem (Shanghai, China). Transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

### *RNA preparation and quantitative real-time polymerase chain reaction*

Total RNA including miRNAs was isolated from tissue or cultured cells using TRIzol® reagent (Invitrogen) according to the manufacturer's instructions, and the concentration of total RNA was determined using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). For quantify miR-138, the cDNA was synthesized using One Step Prime script miRNA cDNA Synthesis Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Then miR-138 was quantified using the QuantMir RT Kit (System Biosciences, Mountain View, CA, USA under ABI 7900 Sequence Detection System (Life Technologies, NY, USA) with miR-138 specific primers as previous described [19]. U6 snRNA was used as an endogenous control. To detect SOX9 expression, the cDNA was synthesized using the Takara PrimeScript™ First Strand cDNA Synthesis kit (Takara Bio, Inc., Dalian, Japan) according to the manufacturer's instructions. The expression of SOX9 mRNA was detected by using Real-time PCR Mixture Reagent (Takara) under ABI 7900 Sequence Detection System with SOX9 specific primers as described previously [20]. Data were collected and analyzed using $2^{-\Delta\Delta Ct}$ method for quantification of the relative miR-138 and SOX9 mRNA expression levels. U6 and GAPDH were used as internal controls for miRNAs and mRNAs, respectively.

### *Cell proliferation and colony formation assay*

Cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In briefly, transfected cells were seeded into 96-well culture plates at a density of 2,000 cells in 200 μl/well, and incubated at 37°C. At indicated time points (24, 48, 72, or 96 hours), 100 μL of MTT solution (0.5 mg/mL, Sigma-Aldrich Corp, St Louis, MO, USA) was added to each well, and were further cultured for another 4 hours. Then, the MTT solution was removed and 150 μl of dimethyl sulfoxide (DMSO, sigma) was added to each well to stop the reaction, followed by measuring the absorbance at 490 nm on an enzyme immunoassay analyzer (Bio-Rad, USA) after the plates were gently shaken on a swing bed for 10 minutes.

For the colony formation assay, transfected cells were resuspended and seeded onto 6-well

plates at a density of 1000 cells/well and cultured in growth media. After 14 days of growth colonies were stained with 0.5% crystal violet for 30 min, examined by light microscopy using an Olympus CK2 phase contrast inverted microscope (Olympus America Inc., Center Valley, PA). The percentage colony formation was calculated by adjusting control to 100%.

*Transwell migration and invasion assays*

Cell migration assay was determined by wound healing assay. In briefly, the $2\times10^4$ transfected cells were seeded into 24-well culture plates. When cells were grown to confluence, an artificial homogeneous wound was created onto the monolayer with a sterile plastic micropipette tip, and cultured under standard conditions for another 24 h. After the debris was removed by washing with PBS, migration of cells into the wound was observed and photographed under an inverted microscope.

Invasive abilities were examined using 24-well Transwell chambers (8 μm; Corning Inc, Corning, NY, USA).In briefly, $2\times10^5$ transfected cells were seeded into the upper chambers, which were covered with 1 mg/mL Matrigel, and DMEM medium containing 10% FBS was used as the chemo-attractant in the lower chamber. Then, 24 hours later, the cells on the upper surface of the membrane were removed by cotton swabs, and the invaded cells were fixed with 95% ethanol, stained with 0.1% crystal violet, and counted at five randomly selected fields under a light microscope (200×, Olympus, Tokyo, Japan).

*Luciferase assay*

The wild type and mutant 3'UTRs of *SOX9* were synthesized from Genechem (Shanghai, China) and subcloned into was cloned into the firefly luciferase reporter psicheck-2 vector (Promega) at the NotI and XhoI sites, and named as Wt-SOX9-3'UTR and Mut-SOX9-3'UTR, respectively. For the luciferase reporter assay, HepG2 cells were seeded onto 24-well plates at 50% confluence before transfection. Then cells were co-transfected with 100 nM miR-NC or miR-138 mimic, 50 ng pRL-TK (Promega, Madison, WI, USA) and 50 ng firefly luciferase reporter containing the wild-type or mutant-type 3'UTR of *SOX9* using Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, luciferase activity was determined using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) and a microplate fluorescence reader (BioTek). Firefly luciferase was used to normalize the Renilla luciferase.

*Western blot analysis*

Total protein from cells or tissues were lysed by RIPA buffer(ProMab Biotechnology, USA). The concentration of total proteins was quantified using a bicinchoninic acid (BCA) protein assay kit (Boster, China). Equal amounts of protein lysates (30 μg each lane) separated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After blocking with 4% dry milk, the membranes were incubated with primary antibodies against SOX9 (Cell Signaling) and GAPDH at 4°C overnight. Then the membrane was further incubated with horseradish peroxidase (HRP)-conjugated corresponding second antibody for 2 h at room temperature. Protein band was visualized with enhanced chemiluminescence (ECL) reagents (Pierce; Thermo Fisher Scientific Inc, Waltham, MA, USA) and exposed on an X-ray film. GAPDH was used as an internal reference for relative quantification.

*Statistical analysis*

The statistical analyses and graphical depiction of data were performed using GraphPad Prism 5.0. software (San Diego, CA, USA) and the SPSS 16.0 software (SPSS, Chicago, IL, USA). All data at least from three independent experiments were expressed as mean ± SD (standard deviation). Statistical differences were determined by ANOVA or Student t test. Pearson product-moment correlation coefficients were used to determine the association between levels of *SOX9* mRNA and the expression of miR-138. A value of $P<0.05$ was considered statistically significant.

## Results

*miR-138 expression is downregulated in HCC tissues and cell lines*

In order to determine the expression levels of miR-138 in HCC, quantitative RT-PCR analysis was performed in 40 pairs of HCC tissues and pair-matched adjacent noncancerous tissues. As shown in **Figure 1A**, miR-138 was significantly downregulated in HCC tissues compared

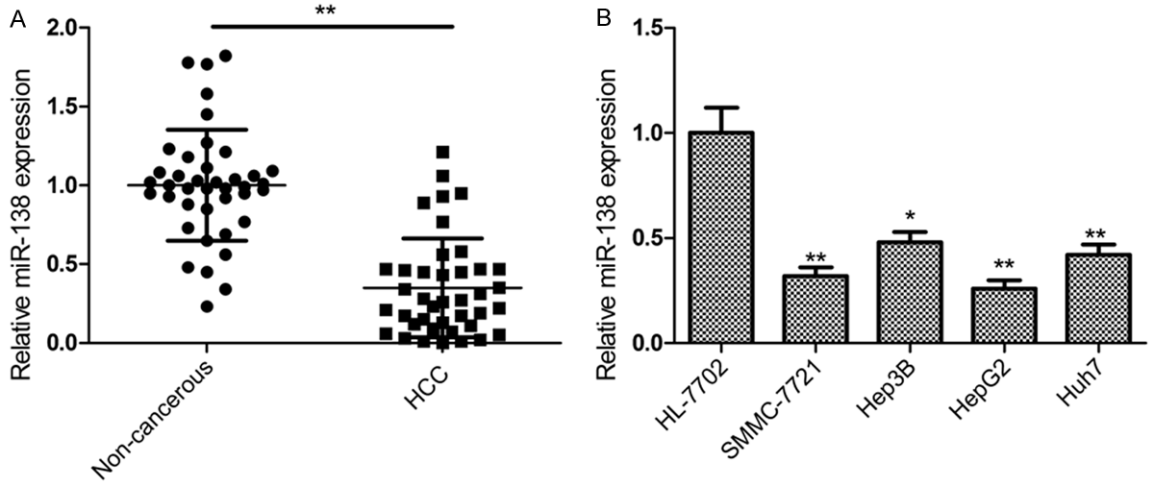


**Figure 1.** miR-138 expression is downregulated in HCC tissues and cell lines. A. qRT-PCR analysis of miR-138 expression in HCC tissues and adjacent no-cancerous tissues from 40 patients with HCC. **P<0.01 versus non-cancerous tissue. B. qRT-PCR analysis of miR-138 expression in four HCC cell lines (SMMC-7721, Hep3B, HepG2, Huh-7) and normal hepatic cell line HL-7702. *P<0.05, **P<0.01 versus HL-7702.

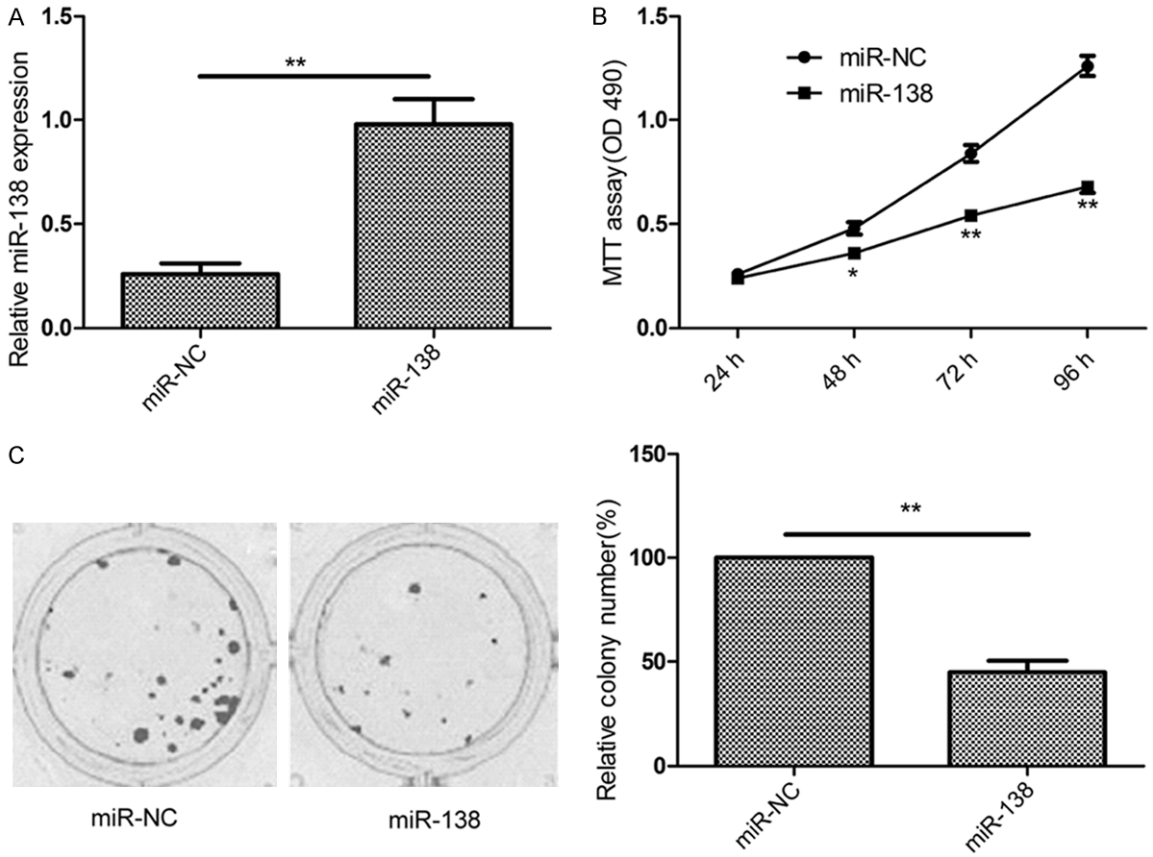


**Figure 2.** miR-138 inhibits cell proliferation and colony formation in HCC cells. A. HepG2 cells were transfected with miR-138 mimics or miR-NC. The expression level of miR-138 was detected by qRT-PCR. B, C. Cell proliferation and colony formation were determined in HepG2 cells transfected with miR-138 mimic or miR-NC. *P<0.05, **P<0.01 versus miR-NC.

with paired normal control tissues. Furthermore, the miR-138 expression was investigated in four HCC cell lines (SMMC-7721, Hep3B, HepG2, Huh-7) and normal hepatic cell line HL-

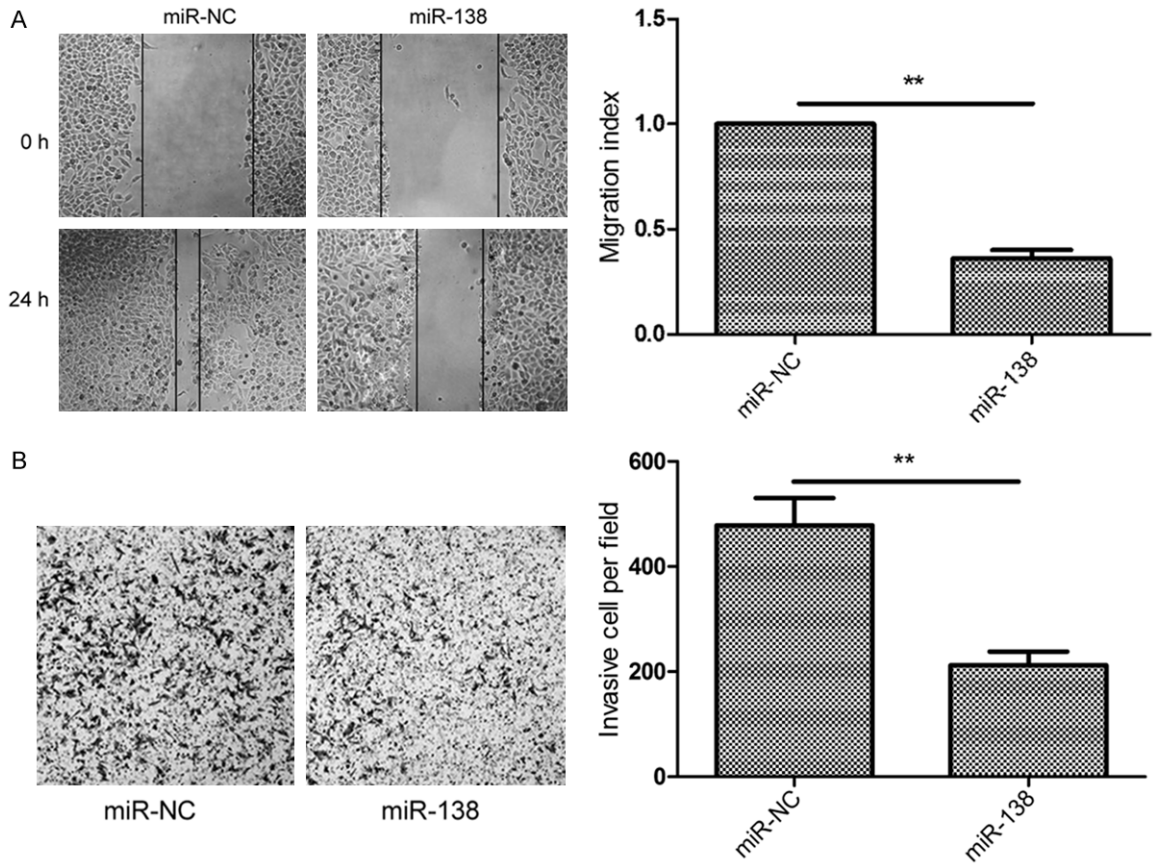


**Figure 3.** miR-138 inhibits cell migration and invasion in HCC cells. A. Wound healing assay for migration in hepG2 cells transfected with miR-138 mimic or miR-NC. B. Transwell assay for invasion in hepG2 cells transfected with miR-138 mimic or miR-NC. *P<0.05, **P<0.01 versus miR-NC.

7702 by qRT-PCR, and results showed that miR-138 expression was significantly decreased in four HCC cell lines compared with normal hepatic cell line HL-7702 (**Figure 1B**). HepG2 displayed a lowest expression level of miR-138 among four cell lines (**Figure 1B**), thus, we selected it as a model for below study.

*miR-138 overexpression inhibits cell proliferation and colony formation in HCC cells*

To assess the function of miR-138 in HCC procession, miR-138 mimic and negative controls (miR-NC) were transfected into HepG2 cells, respectively, and found that miR-138 expression significantly increased in HepG2 cells transfected miR-138 mimic compared to cells transfected miR-NC (**Figure 2A**). We evaluated the effect of miR-138 on cell proliferation using MTT assay. As shown in **Figure 2B**, overexpression of miR-138 significantly inhibited cell proliferation in HepG2 cells. Consistent with this result, our results also showed that overexpression of miR-138 significantly inhibited cell colony formation in HepG2 cells (**Figure 2C**).

*miR-138 overexpression inhibits cell migration and invasion in HCC cells*

Next, we investigated the effect of miR-138 on HCC cell migration and invasion by wound healing and transwell invasion assays, respectively. It was found that overexpression of miR-138 significantly inhibited he migratory and invasive abilities of HepG2 cells (**Figure 3A** and **3B**). These results suggested that miR-138 suppressed the metastasis of HCC cells.

*miR-138 binds to 3'UTR of SOX9 and decreases expression of SOX9 in HCC cells*

In order to explore the mechanism underlying inhibitory effects of miR-138 on proliferation, migration and invasion, we identified target of miR-138 using two bioinformatics software (miRWalk and targetscan6.2). SOX9 is the candidate with higher scores and miR-138 binding site in their 3'UTR (**Figure 4A**). To confirm SOX9 as a direct target of miR-138, we performed luciferase reporter assay in HepG2 cells. As

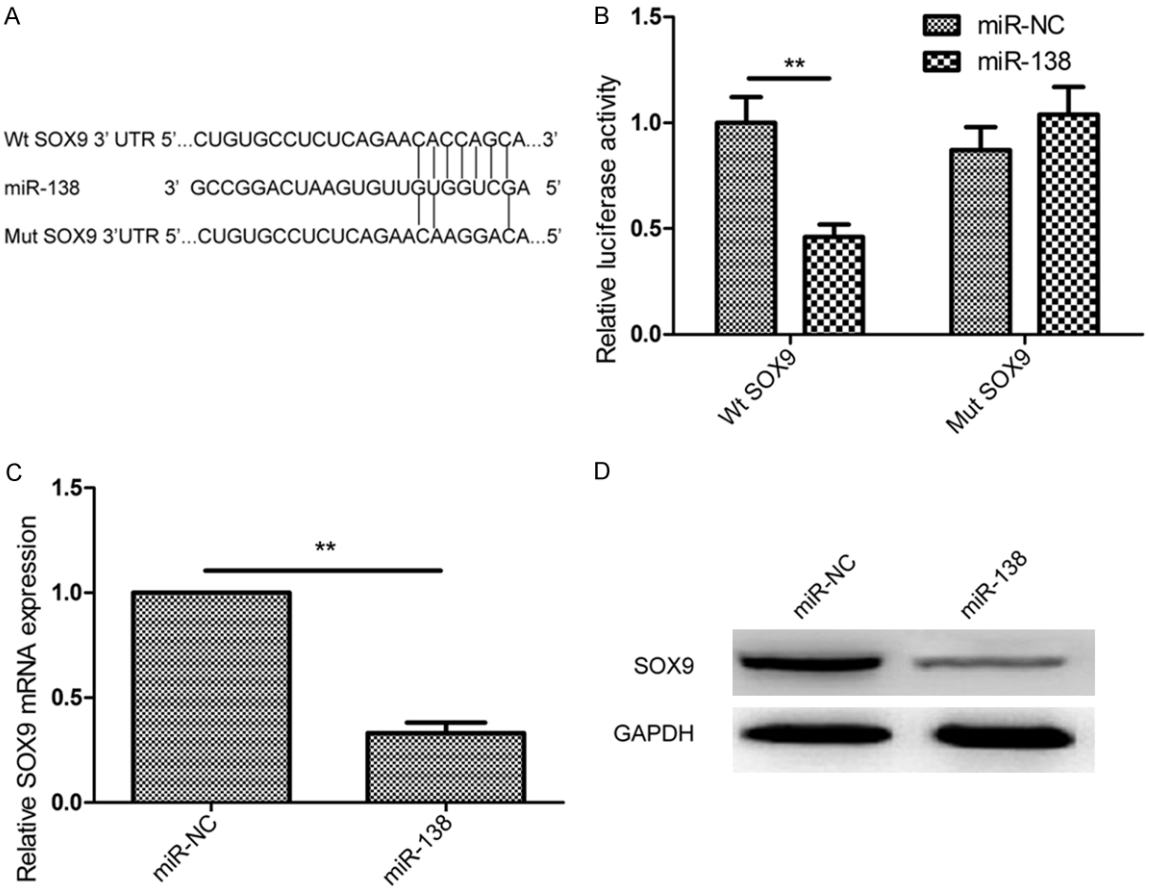


**Figure 4.** miR-138 binds to 3'UTR of SOX9 and decreases expression of SOX9 in HCC cells. A. The potential miR-138 binding sequence of SOX9 3'UTR and the mutant was shown. B. HepG2 cells were co-transfected with miR-138 mimic or miR-NC and Wt or Mut SOX9 3'UTR report plasmid. Luciferase activity was measured. C, D. SOX9 expression on mRNA level and protein level were detected in HepG2 cells transfected with miR-138 mimics or miR-NC. GAPDH was used as a control. *P<0.05, **P<0.01 versus miR-NC.

shown in **Figure 4B**, overexpression of miR-138 caused a significant decrease in luciferase activity in HepG2 cells transfected with the reporter plasmid with wild type targeting sequence of SOX9 mRNA but not reporter plasmid with mutant sequence of SOX9. In addition, our results also showed that overexpression of miR-138 obviously suppressed SOX9 expression on mRNA level and protein level (**Figure 4C** and **4D**). These results suggest that *SOX9* is a target gene of miR-138 in HCC cells.

*SOX9 expression was upregulated and inversely correlated with miR-138 expression in HCC tissues*

Knowing SOX9 was the target of miR-138, we detected it in the CRC tissue samples and adjacent non-tumor tissues. We found that SOX9 expression on mRNA level (**Figure 5A**) and protein level (**Figure 5B**) was greatly increased in HCC tissues compared with adjacent non-cancerous tissues. Meanwhile, the miR-138 and SOX9 correlation were also investigated in CRC tissues. Pearson product-moment correlation coefficients analysis showed a reversed correlation between miR-138 expression levels and SOX9 mRNA levels in HCC tissues (**Figure 5C**, $r$=-0.689, $P$<0.01). In addition, SOX9 mRNA expression was also investigated in four HCC cell lines (SMMC-7721, Hep3B, HepG2, Huh-7) and normal hepatic cell line HL-7702 by qRT-PCR, and found that miR-138 expression was significantly increased in four HCC cell lines compared with normal hepatic cell line HL-7702 (**Figure 5D**).

*Decreased expression of SOX9 showed similar effect with miR-138 overexpression in HCC cells*

The above results prompted us to examine whether the suppressive effect of miR-138 is regulated by repression of SOX9 in HCC cells.

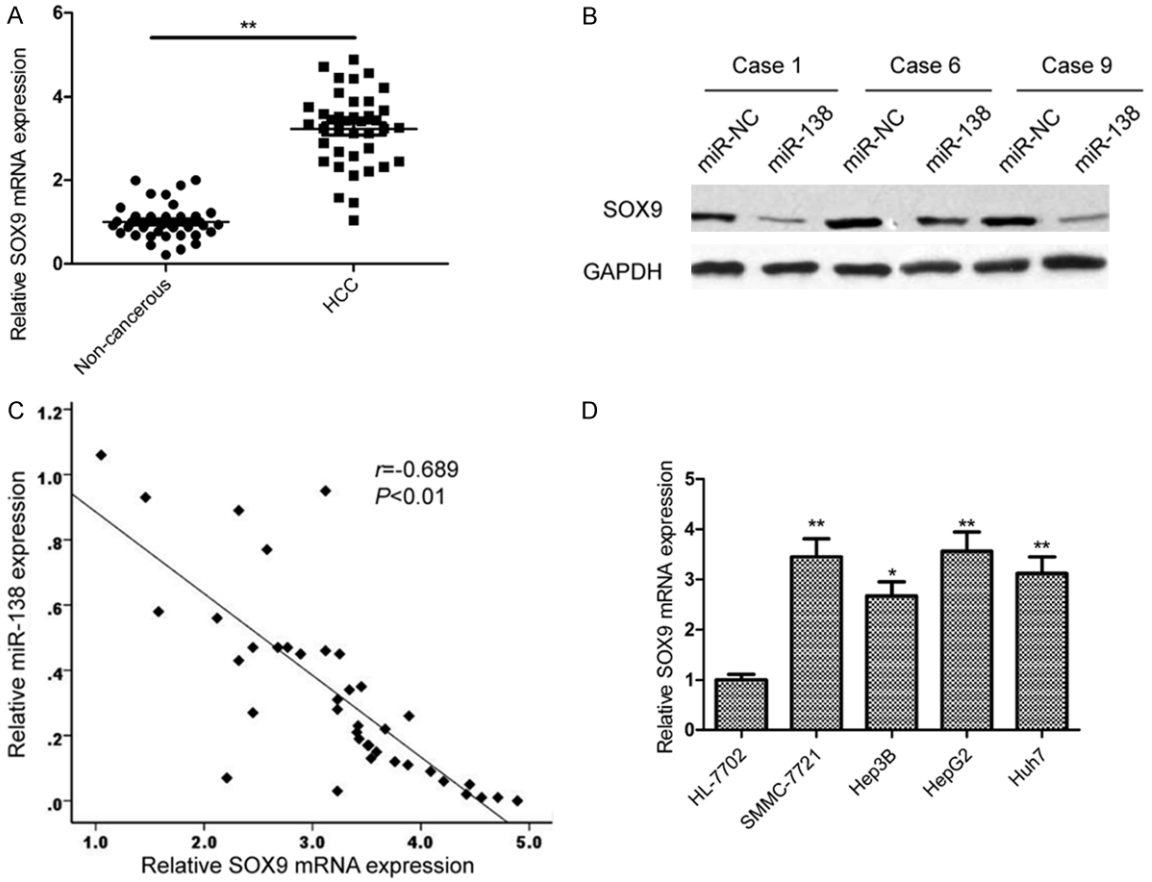


**Figure 5.** SOX9 expression was upregulated and inversely correlated with miR-138 expression in HCC tissues. A, B. SOX9 expression on mRNA level and protein level were detected HCC tissues and adjacent no-cancerous tissues. GAPDH was used as a control. *P<0.05, **P<0.01 versus **P<0.01 versus non-cancerous. C. The reverse relationship between SOX9 and miR-138 expression was explored in HCC tissues by pearson product-moment correlation coefficients assay. D. qRT-PCR analysis of SOX9 mRNA expression in four HCC cell lines (SMMC-7721, Hep3B, HepG2, Huh-7) and normal hepatic cell line HL-7702. *P<0.05, **P<0.01 versus HL-7702.

Therefore, HepG2 cells were transfected with si-SOX9 and si-NC, and SOX9 expression was significantly decreased in HepG2 cells transfected with si-SOX9 compared to cells transfected with miR-NC (**Figure 6A** and **6B**). Inhibition of SOX9 by siRNA significantly suppressed the proliferation, colony formation migration and invasion of HepG2 cells (**Figure 6C-F**), similar to those induced by miR-138. Taken together, these findings indicated that SOX9 is a functionally important target of miR-138 in HCC cells.

## Discussion

In the present study, we found that miR-138 expression was downregulated in HCC cell lines and in primary tumor samples, which was in consistent with previous study [21]. Moreover, *in vitro* functional assays demonstrated that the overexpression of miR-138 expression inhibited cell proliferation, colony formation, migration and invasion in HCC cells. Finally, SOX9 was identified as a direct target of miR-138, and downregulation of SOX9 expression has similar effect with miR-138 overexpression in HCC cells. To our knowledge, this study is the first to show that miR-138 inhibited cell proliferation, migration and invasion in HCC cells by targeting SOX9.

MicroRNA 138 (miR-138) has been shown to involved in various biological processes, such as embryological morphogenesis, cell proliferation, cell invasion and developmental events tied to stem cell differentiation [22]. In multiple cancers, miR-138 has been shown to be downregulated and to plays an important role in tumor procession and development, and to serve as a tumor suppressor [11-17]. For exam-

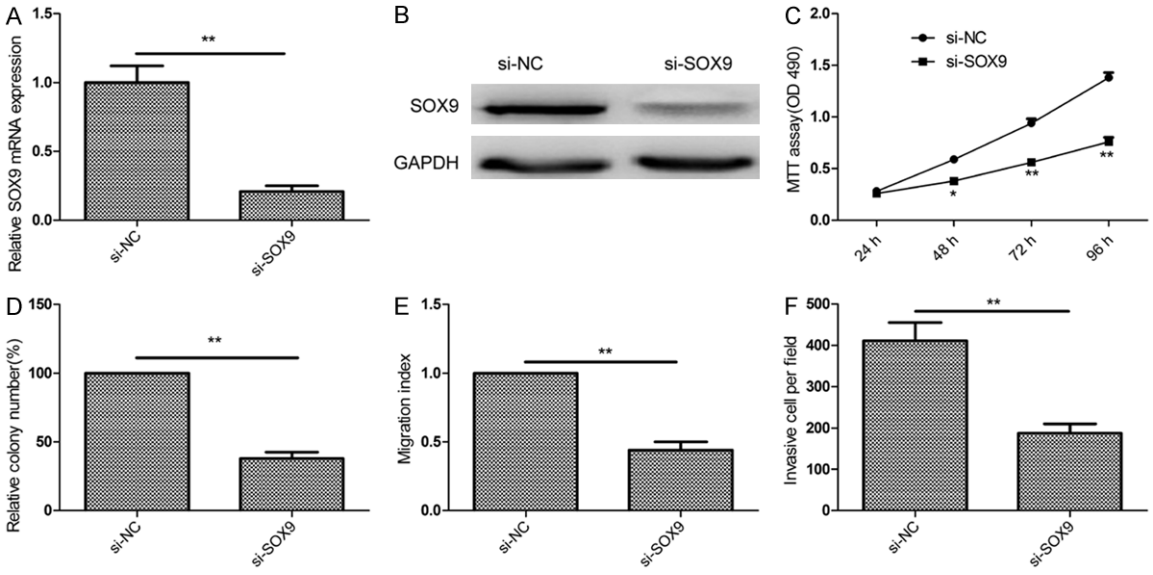


**Figure 6.** Decreased expression of SOX9 showed similar effect with miR-138 overexpression in HCC cells. A, B. SOX9 expression on mRNA level and protein level was determined in HepG2 cells transfected with si-SOX9 or si-NC. GAPDH was used to an internal control. C-F. Cell proliferation, colony formation, migration and invasion were determined in HepG2 cells transfected with si-SOX9 or si-NC. *P<0.05, **P<0.01 versus miR-NC.

ple, Mitomo *et al.* reported that downregulation of miR-138 was associated with overexpression of human telomerase reverse transcriptase (hTERT) protein in human anaplastic thyroid carcinoma cell lines, which contribute to promote anaplastic thyroid carcinoma cell proliferation and invasion [23]. Ma *et al.* found that miR-138 suppresses gallbladder carcinoma cells growth and induces apoptosis by directly targeting Bag-1 [15]. Zhang et al. demonstrated that miR-138 inhibited tumor growth through repression of EZH2 in non-small cell lung cancer [24]. For HCC, although a report showed that miR-138 induced cell cycle arrest in hepatocellular carcinoma by targeting cyclin D3 [18], the detail biological function, especially in regards to affect migration and invasion, and underlying molecular mechanism of miR-138 in HCC has not been total elucidated. Here, we found that miR-138 inhibited cell proliferation, colony formation, migration and invasion in HCC cells partially repressing SOX9 expression. Our results combined with previous report suggested that miR-138 functioned as tumor suppressor in HCC by targeting multiple genes.

To elucidate the anti-tumor mechanism of miR-138 in HCC, its target genes were investigated using two bioinformatics software (miRWalk and targetscan6.2). SOX9 (sex determining region Y [SRY] related highmobility group box 9), a member of the SRY box gene superfamily [25], were selected as study object. As a transcriptional regulator, SOX9 has been showed to involve in various biological process, such as chondrogenesis, male sex gonad or respiratory epithelium development, melanocyte differentiation, and the differentiation of Paneth cells in the gut [26, 27]. It has been showed that SOX9 was usually overexpressed in many type cancers, and function as oncogene [28-30]. Accumulating evidence suggested that SOX9 promoted tumor cell growth and metastasis, at least in part, through regulation of the Wnt pathway, Notch1 pathway, P53 pathway, and epithelial-tomesenchymal transition (EMT) [31-33]. In addition, it has been demonstrated that SOX9 expression was upregulated in HCC tissues, and its expression was significantly associated with advanced tumor/nodes/metastasis (TNM) classification stages, and shorter overall survival [34], which suggested that SOX9 functioned as oncogene in HCC. In the present study, we confirmed that SOX9 is a direct target gene of miR-138 in HCC by luciferase reporter assay, qRT-PCR and western blot. We also found that SOX9 expression was upregulated in HCC cells and tissues, and its mRNA expression was reverse correlated with miR-138 expression in HCC tissues. Of note, downregulation of SOX9 expression inhibited cell proliferation, colony formation, migration and invasion in HCC cells, yielding very much similar effect as that of miR-138. These results suggested

that miR-138 exerted it anti-tumor role in HCC, at least in part, by targeting SOX9.

In summary, the present study showed that miR-138 was downregulated in HCC cell tissues and cell lines, and its ectopic expression inhibited cell proliferation, colony formation, migration and invasion in HCC cells. In addition, our results showed that SOX9 is a target gene of miR-138 in HCC cells, and that miR-138 exerts its tumor-suppressor function in HCC cells by repressing SOX9 expression. These results suggest that miR-138 may be a potential therapeutic target for the treatment of HCC.

## Disclosure of conflict of interest

None.

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