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Original Article

MicroRNA-138 suppresses cell proliferation, migration and invasion by targeting smoothened (SMO) in hepatocellular carcinoma

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Abstract: Hedgehog (Hh) signaling pathway is a crucial signal transduction pathway that associated with tumorigenesis in multiple human tissues. Recently, microRNA-138 (miR-138) has been reported to be frequently involved in different tumorigenesis, including hepatocellular carcinoma (HCC). However, whether miR-138 was correlated with Hedgehog (Hh) signaling pathway implicated in the progression of HCC remains unclear. Here, we demonstrated that the expression of miR-138 was downregulated in HCC tissues compared with their matched adjacent non-tumor tissues ($P < 0.001$). Overexpression of miR-138 leaded to impaired cell viability, colony formation, cell migration and invasion of HepG2 cells. Furthermore, smoothened (SMO) was identified as a novel target of miR-138, and the expression of SMO was inhibited by miR-138 overexpression in HepG2 cells. Besides, SMO was upregulated in HCC tissues compared to the matched adjacent normal tissues and its downregulation photocopied the effects of miR-138 overexpression in HepG2 cells. These findings indicate that miR-138 functioned as a tumor suppressor partially via repressing SMO expression in HCC.

Keywords: Hepatocellular carcinoma, miR-138, SMO, cell proliferation, invasion

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancer and the third leading causes of cancer-related death, which is mainly associated with hepatitis C and/or B virus infection [1, 2]. HCC incidence rates are rapidly increasing, and about 20,000 new cases were predicted to occur in United States [3]. Most patients have poor prognosis and high recurrence rate due to easily occurred intrahepatic and extrahepatic metastases accidents [4]. Intensive study has led to a deeper understanding risk factors and pathogenesis of HCC, but the viable strategy options against invasion and metastasis are lacking and the treatment still relies on surgical resection, radiotherapy, chemotherapy [5-7]. Thus, there is an urgent need to develop an effective therapeutic target for HCC.

MicroRNAs (miRs) are small non-coding RNAs with nucleotides length ranging from 19 to 24 [8]. They function as guide molecules in mRNA degradation and post-transcriptional expression [9]. MiRs play critical roles across nearly all development and pathological processes, particularly in cancer [10, 11]. Surprisingly, compared with normal tissues, miRs expression are universally downregulated in human cancer, indicating that miRNAs has the potential to act as tumor suppressors and oncogenes [11]. Currently, several miRs are reported to be involved in inhibition of tumor growth and metastases, such as miR-34a, miR-31, and miR-451, *etc* [12-14]. MiR-138 belongs to miRNA family and is significantly down-regulated in diverse cancer types. For example, miR-138 and miR-26a play an anti-proliferative role by blocking the G1/S transition through a concerted targeting of cell cycle regulating network in prostate cancer [15]. MiR-138 induces cell cycle arrest at G1/S by targeting cyclin D3 in HCC [16]. Overexpression of miR-138 in human ovarian cancer cells is associated with inhibition of invasion and metastasis via targeting SOX4 and HIF-1α [17].

Hedgehog (Hh) signaling pathway is one of the most important signal transduction pathways and crucial to tumorigenesis in multiple human tissues [18]. Hh signaling pathway occurs via two multi-pass membrane-spanning proteins Patched (Ptc) in Hh binding and Smoothened (SMO) in transducing signal [19]. When Hh binding to Ptc of the Smo-Ptc complex, the inhibition of Smo by Ptch is released and glioma-associated (Gli) translocation to the nucleus is promoted. Subsequently, this process transactivates downstream target genes associated with cell cycle progression, apoptosis, stem cell differentiation, *etc* [20]. Previous study found that miR has the potential to regulate Hh pathway to affect tumor development. MiR-125b, miR-326 and miR-324-5p inhibit medulloblastoma cells growth through targeting Hh signaling pathway components (SMO and Gli) [21]. MiR-212 facilitates pancreatic cancer progression and metastasis though targeting Ptc-1 [22]. MiR-14 was identified as an essential modulator of Hh signaling activity [23]. These studies indicate miR regulation plays a critical role of in the Hh pathway. MiR-138, a member of the miRNA family, has been found to be implicate in tumorigenesis and development, however, whether there is an association between miR-138 and Hg signaling pathway are unknown.

In this study, the expression and function of miR-138 in HCC, and its relationship with SMO were explored. Surprisingly, SMO was found to be a direct target of miR-138 in HCC cells. Overexpression of miR-138 inhibited tumor cell proliferation, colony formation, migration, and invasion by directly targeting SMO in HCC. Our findings will facilitate the development of target genes and therapeutic opportunities for HCC.

Materials and methods

Tissue samples collection

Total 20 pairs of HCC tissues and their matched adjacent non-tumor tissues were collected from HCC patients who underwent surgery at Yantai Hospital for Infectious Diseases (Shandong, China) from March 2016 to August 2016. All specimens were histologically and clinically diagnosed, and then immediately frozen in liquid nitrogen after resection and stored at -80°C before use. The written informed consent was obtained from all patients. This study was approved by the Ethics Committee of Yantai Hospital for Infectious Diseases, Shandong, China.

Cell lines and transfection

Human HCC cell lines (HepG2, SMMC-7721, Huh7 and Hep3B) and a human immortalized normal hepatocyte cell line LO2 were purchased from the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) with 10% fetal bovine serum (FBS, Gibco, USA) and anti-biotics (Sigma-Aldrich, MO, USA). Cell cultures were incubated in a humidified incubator containing 5% CO_2 at 37°C.

The miR-138, small interfering RNA against SMO (siSMO) and their corresponding negative control (NC) were designed and synthesized by GenePharma (Shanghai, China). The above vectors were transiently transfected into HepG2 cells using Lipofectamine 2000 (Life Technologies) in accordance with the manufacturer's instruction. The cells without transfection with any vector were used as blank control. Transfected cells were then cultured for 48 h before the following assays.

Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted from tissues and cultured cells using Trizol reagent (Life Technologies) according to the manufacturer's instruction. The cDNAs were synthesized from total RNA using Takara PrimeScript™ First Strand cDNA Synthesis kit (Takara Bio, Inc., Japan). The sequences of the primers were as follows: miR-138 forward, 5'-AGCTGGTGTTGTGAATCAGGCCG-3' and miR-138 reverse, 5'-TGGTGTCGTGGAGTCG-3'; U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and U6 reverse, 5'-AACGCTTCACGAATTTGCGT-3'; SMO forward, 5'-TAGCCCTGCGTAGCCAGTTA-3' and SMO reverse, 5'-TCATGCTTAGTCCACTGTCTGT-3'; GAPDH forward, 5'-ACAACTTTGGTATCGTGGAAGG-3' and GAPDH reverse, 5'-GCCATCACGCCACAGTTTC-3'. The relative expression levels of miR-138 and SMO were detected by qRT-PCR using TaqMan miRNA assay (Ambion, CA, USA) and standard SYBR Green RT-PCR kit (Takara, Japan), respectively in accordance with the manufacturer's instruction. The $2^{-\Delta\Delta Ct}$ method was used to quantify the relative expression level. U6 and

GAPDH were used as an internal reference for miR-138 and SMO, respectively.

Western blot analysis

Tissues or cells were harvested and lysed in ice-cold RIPA buffer (Life Technologies) to extract proteins following the manufacturer's protocol. The protein concentration was determined using the BCA protein assay kit (Boster, China). Equal amounts of protein lysates were separated with 10% SDS-PAGE and then transferred to a PVDF membrane (EMD Millipore, USA). After blocking with 5% nonfat dried milk for 4 h, the membrane was then incubated with primary antibodies against SMO (1: 500, Abcam, USA) and GAPDH (1: 5000, Cell Signaling Technology, USA) at 4°C overnight. GAPDH was used as the internal control. Membranes were washed with TBST three times and further incubated with horseradish peroxidase-conjugated corresponding second antibody (Santa Cruz Biotechnology, USA) for 2 h at room temperature. After washing by TBST, Protein signal was detected with enhanced chemiluminescence (ECL) Western Blotting Kit (Pierce, MA, USA).

CCK-8 assay

The cell viability was assessed by cell counting Kit-8 (CCK-8; Peninsula Labs, Belmont, CA, USA). Briefly, transfected cells were seeded in 96-well plates at a density of 3×10^4 cells per well and cultured in DMEM medium containing 10% FBS for 24 h. The cell viability was detected by adding 5 μl of CCK-8 solution to each well, and the plates were further incubated for 2 h. Then the optical density (OD) value at 450 nm was measured by using an ELISA reader every 24 h. Each sample was analyzed three times.

Colony formation assay

Stable transfected cells were seeded in 6-well plates at a density of 300 cells per well. After 8 days of culture, colonies were naturally formed. The cells were washed twice with phosphate buffered saline (PBS) carefully, and then fixed with 4% paraformaldehyde for 15 min. The fixed cells were stained with crystal violet for 20 min. Finally, the colony formation was observed and colonies (each colony containing more than 50 cells) were counted through a light microscope.

Transwell assay

Transwell migration assay was performed to assess cell migration. Briefly, transfected cells (5×10^4 cells per well) were seeded in serum-free media in the upper Transwell chambers (BD Biosciences) and incubated overnight to allow the cells to attach. The lower chamber was added complete media as a chemoattractant. After 48 h incubation, cells that migrated to the lower surface of the filter were fixed in 70% ethanol solution (Sigma-Aldrich) for 30 min and stained with 2% crystal violet solution for 10 min. The migrated cells were photographed and counted in 5 randomly selected fields under and inverted microscope. Similarly, the invasion assay was performed except the upper chamber contains Matrigel-coated membrane.

Dual luciferase reporter assay

In brief, the wild type (WT) and mutant (MUT) 3'UTRs of SMO containing putative binding site of miR-138 were synthesized from Genechem (Shanghai, China) and inserted into the Firefly luciferase expressing psiCHECKTM-2 vector (Promega, Madison, USA) in accordance with the manufacture's protocol. For the luciferase reporter assay, HepG2 cells were seeded into 24-well plates and cultured to approximately 80% confluence. Then cells were transfected with either WT or MUT plasmid (100 ng) and co-transfected with NC or miR-138 mimic (50 nM) using Lipofectamine 2000 (Invitrogen). After 48 h, cells were harvested and the activities of renilla luciferase and firefly luciferase were determined using dual-luciferase reporter assay system (Promega). The relative luciferase activity was calculated as the ratio of firefly luciferase activity value and renilla luciferase activity.

Statistical analysis

All experiments were performed three times and all quantitative data were expressed as mean ± SD. Statistical analysis of differences was performed by student's t test using SPSS 17.0 software. Differences were considered significant at p value less than 0.05.

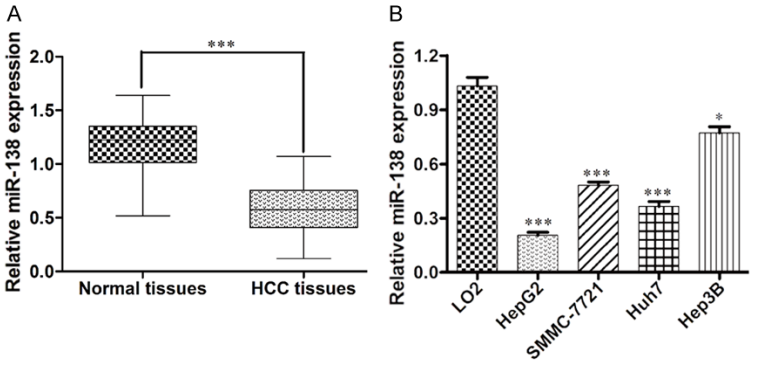

Figure 1. The expression of miR-138 is downregulated in HCC tissues and cell lines. A: qRT-PCR was performed to analyze the expression levels of miR-138 in 20 cases of HCC and their matched adjacent non-tumor tissues. ***P < 0.001 versus non-tumor tissues; B: qRT-PCR was used to determine the expression levels of miR-138 in HCC cell lines (HepG2, SMMC-7721, Huh7 and Hep3B) and normal hepatic cell line LO2. GAPDH was used as an internal control. *P < 0.05, ***P < 0.001 versus LO2.

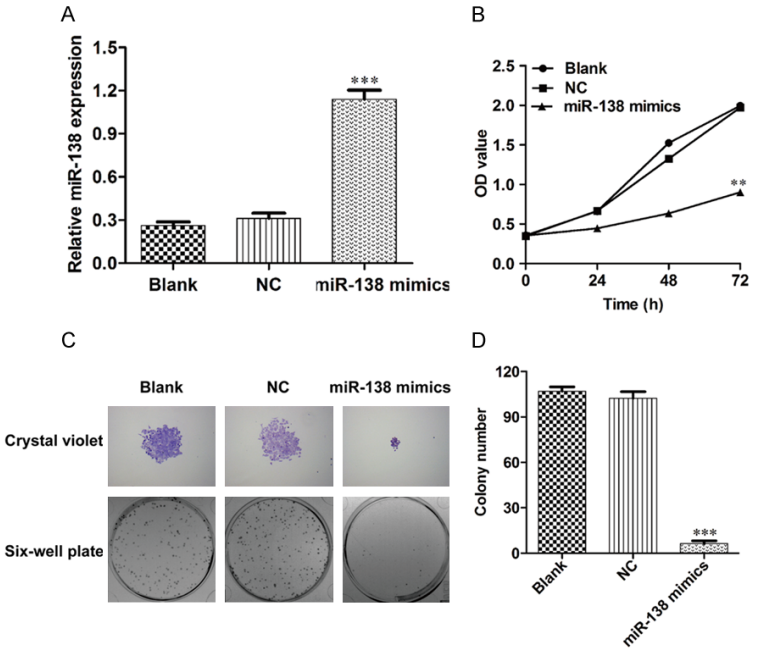

Figure 2. Overexpression of miR-138 inhibits cell viability and colony formation in HCC cells. A: qRT-PCR was used to detect the expression level of miR-138 in HepG2 cells after transfected with miR-138 mimics or NC. B: Cell viability was determined in HepG2 cells following miR-138 mimics or NC transfection using CCK-8 assay. C: Representative images of single colony and total colonies in plates were shown in HepG2 cells following miR-138 mimics or NC transfection. D: The average number of colonies in each plate was shown in the histogram. **P < 0.01, ***P < 0.001 versus NC or Blank.

Results

MiR-138 was downregulated in HCC tissues and cell lines

To investigate the role of miR-138 in HCC, total 20 pairs of HCC tissues and their matched adjacent non-tumor tissues were used to analyze its expression pattern by qRT-PCR analysis. As shown in **Figure 1A**, the expression of miR-138 was significantly downregulated in HCC tissues compared with adjacent non-tumor tissues (P < 0.001). In cell level, miR-138 expression was also observed to be lower in several HCC cell lines (HepG2, SMMC-7721, Huh7 and Hep3B) than that in the normal liver cell lines LO2 (**Figure 1B**, P < 0.05, P < 0.001). Among these HCC cell lines, the expression of miR-138 was the lowest in HepG2 cells. These data indicated that miR-138 was downregulated in HCC.

Upregulation of miR-138 inhibited HCC cell viability and colony formation ability

To examine the function roles of miR-138 in HCC, HepG2 cells with the lowest miR-138 were used to conduct gain-of-function experiments. HepG2 cells were transfected with miR-138 mimics or NC and then miR-138 level was examined using qRT-PCR analysis. As illustrated in **Figure 2A**, cells transfected with miR-138 mimics showed a significant increase in miR-138 level compared with transfected with NC or Blank group (P < 0.001). Then CCK-8 and colony formation assays were performed to detect cell proliferation ability. As shown in **Figure 2B**, overexpression of miR-138 significantly inhibited cell viability in HepG2 cells (P < 0.01). Consistent with this result, colony formation assay also demonstrated that overexpression of miR-138 led to a remarkable reduction of colony number in HCC cells (**Figure 2C** and **2D**, P < 0.001). These results proved that miR-138 has a suppressive effect on HCC cell growth.

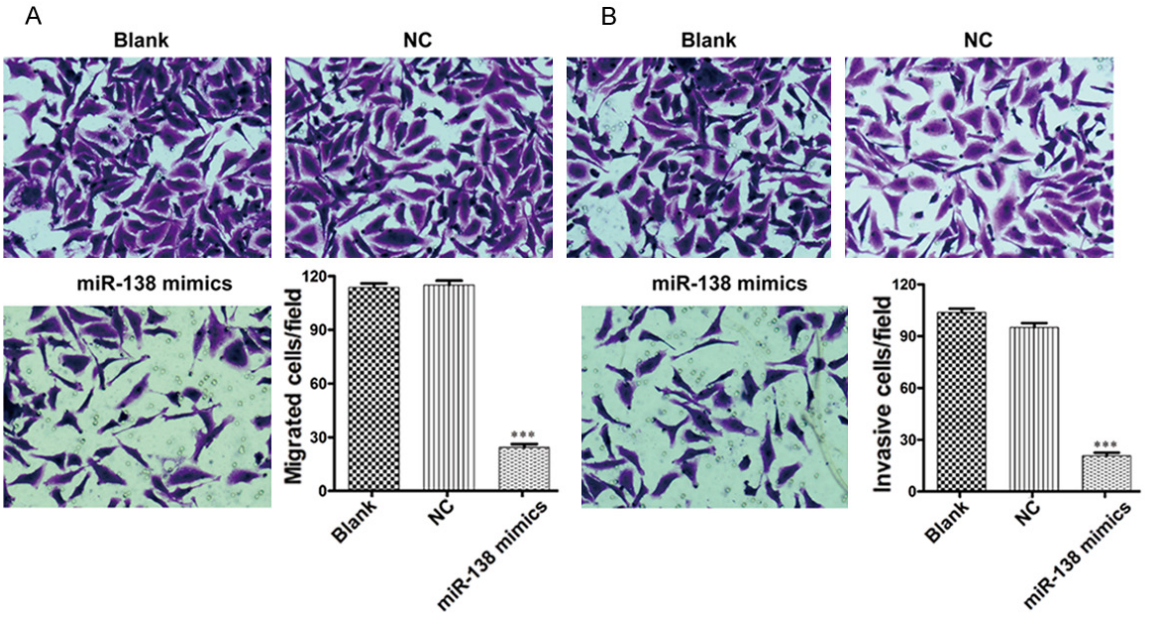

Figure 3. Overexpression of miR-138 decreases cell migration and invasion ability in HCC cells. (A) Cell migration and (B) invasion ability were assessed in HepG2 cells transfected with miR-138 mimics or NC using Transwell assay, respectively. ***P < 0.001 versus NC or Blank.

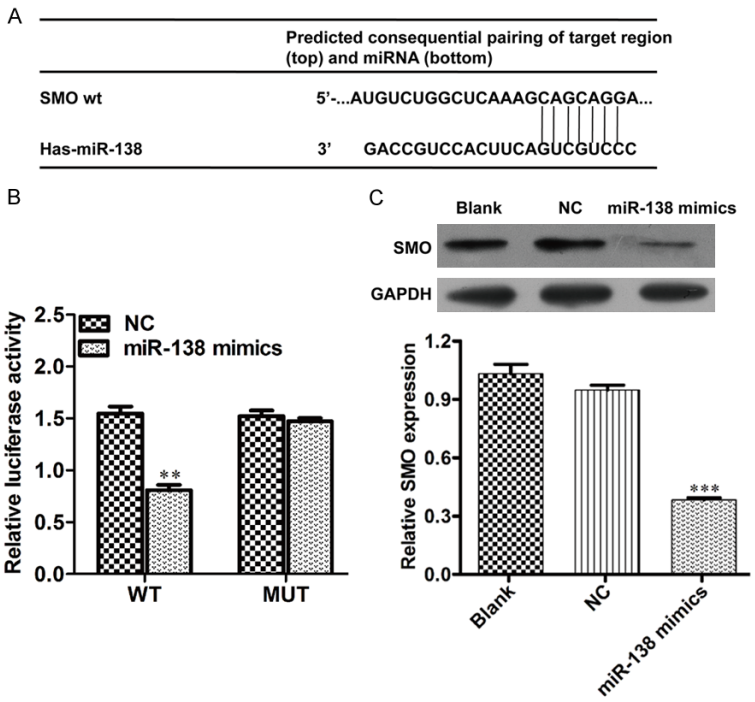

Figure 4. SMO is a direct target of miR-138 in HCC. A: The predicted binding sequence of miR-138 and candidate target gene SMO 3'UTR by TargetScan. B: HepG2 cells were co-transfected with wildtype or mutant SMO 3'UTR constructs and miR-138 mimics or NC. Luciferase activity was measured. C: The expression of SMO at mRNA and protein levels were detected in HepG2 cells transfected with miR-138 mimics or NC. GAPDH was used as an internal control. **P < 0.01, ***P < 0.001 versus NC or Blank.

Upregulation of miR-138 repressed migration and invasion of HCC cells

Next, we investigated the function of miR-138 in cell migration and invasion using Transwell chamber assay in HCC cells. As shown in **Figure 3A**, the number of migrated cells in miR-138 overexpression group (25 ± 3) was significantly decreased, compared with the NC (115 ± 6) or Blank group (112 ± 4) in HepG2 cells (P < 0.001). The similar results were also observed in invasion assay in HepG2 cells after miR-138 overexpression (**Figure 3B**, P < 0.001). These findings further indicated that miR-138 plays an inhibitory role in HCC cell metastasis.

SMO is a direct target of miR-138 in HCC cells

It has been reported that miR achieves its biological function by regulating its target ge-

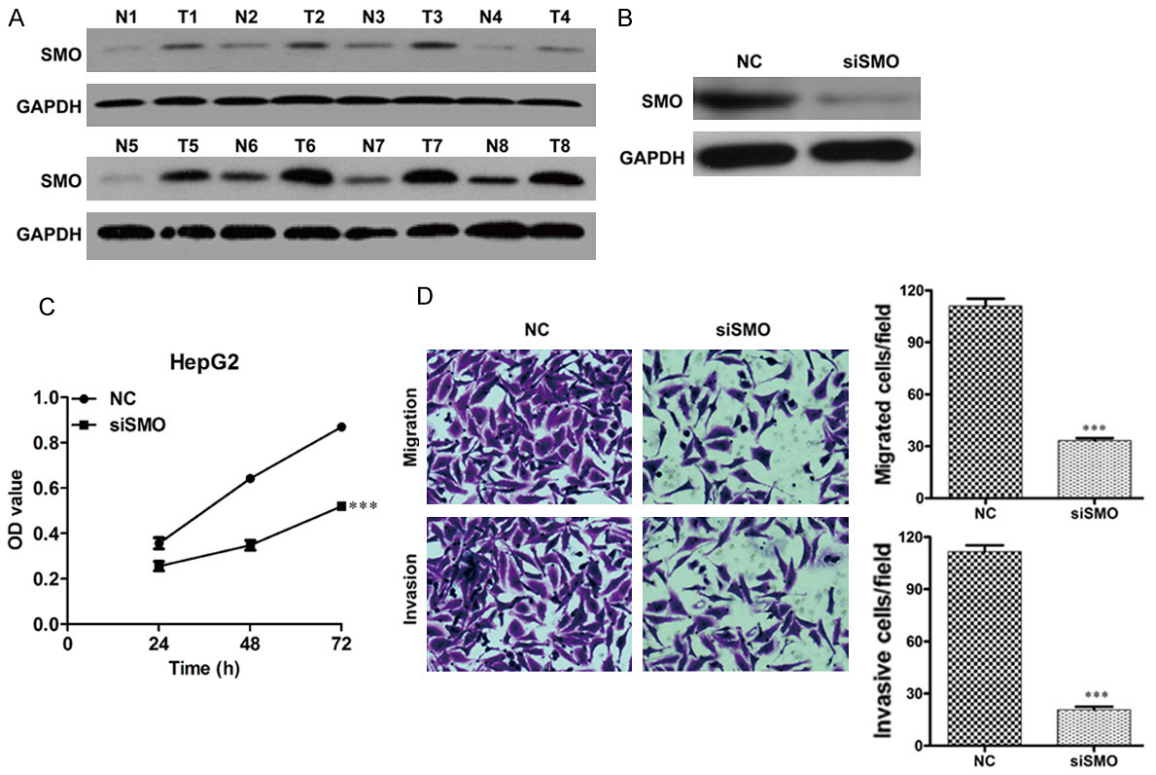

Figure 5. Downregulation of SMO shows similar effects to miR-138 overexpression in HCC cells. (A) Western blotting was performed to analyze the protein levels of SMO in 8 cases of HCC and their matched adjacent non-tumor tissues. (B) SMO expression at protein level was determined in HepG2 cells transfected with siSMO or NC. GAPDH was used as an internal control. (C) Cell proliferation, (D) migration and invasion were determined in HepG2 cells transfected with siSMO or NC. ***$P < 0.001$ versus NC.

nes [24]. Using TargetScan predication, SMO was identified as one of the target genes that was predicted to binding miR-138 (**Figure 4A**) and previously reported to be related to HCC biological progress [25]. To verify this bioinformatics predication, a dual-luciferase reporter system was carried out to determine whether SMO was a direct target of miR-138. As shown in **Figure 4B**, overexpression of miR-138 significantly downregulated the luciferase activity of the wild type SMO 3'UTR, but failed to affect the mutant 3'UTR in HepG2 cells ($P < 0.01$). Consistently, overexpression of miR-138 decreased the expression of SMO at both the mRNA and protein levels in HepG2 cells (**Figure 4C**, $P < 0.001$). Based on these data, we could conclude that SMO was a direct target of miR-138 in HCC cells.

Downregulation of SMO showed suppressive effects on HCC cell proliferation, migration and invasion

The above results prompted us to investigate the role of SMO in HCC cells. The expression of SMO was firstly determined in 8 pairs of HCC tissues and adjacent non-tumor tissues using Western blotting. As shown in **Figure 5A**, SMO expression was obviously upregulated in HCC tissues compared with non-tumor tissues. Then, HepG2 cells were transfected with siSMO or NC and SMO was significantly downregulated in siSMO transfected cells compared with NC (**Figure 5B**). The further results revealed that downregulation of SMO expression inhibited proliferation, migration and invasion in HepG2 cells (**Figure 5C** and **5D**, $P < 0.001$), which was similar to the effect of miR-138 overexpression on proliferation, migration and invasion of HepG2 cells. These results demonstrated that miR-138 inhibited the proliferation, migration and invasion of HCC cells by targeting SMO.

Discussion

HCC is a clinical challenge disease with higher incidence rate, but lack of effective treatment options for against invasion and metastasis. In this study, miR-138 was found down-regulated in HCC tissues and HCC-derived cell lines.

Excessive expression of miR-138 leaded to block and inhibition of proliferation, colony formation, invasion, and migration in HCC cells. Moreover, SMO was identified as a direct downstream target of miR-138 and knockdown of SMO inhibited cell proliferation and metastasis in HCC. In brief, the results of our study demonstrate that up-regulation of miR-138 protected against the development of HCC possibly by partially targeting SMO. The identification and characterization of miR-138 and its relationship with SMO may thus provide a novel therapeutic strategy for treating HCC.

Previous reports showed that miR-138 functions as anti-oncogene and inhibits tumorigenesis, including HCC, leukemia and nasppharyngeal carcinoma [16, 26, 27] by targeting genes, such as human telomerase reverse transcriptase protein, cyclin D3, and cyclin D1. In the present study, the SMO protein showed reduced expression in miR-138 overexpressed HepG2 cells, and the binding specificity between miR-138 and SMO 3'UTR were confirmed by TargetScan prediction and luciferase reporter assays. These results indicate that SMO is a direct target of miR-138. Thus, miR-138 has the potential to regulate multiple target genes and may participate in a variety of signaling events.

Hh pathway is a signaling cascade that usually deregulated in multiple tumor types and implicated in their pathological consequences [28]. Previous study found that blockage of Hh pathway by cyclopamine lead to inhibition of proliferation and induction of apoptosis in HCC cells [29]. Abnormal activation of the Hh signaling pathway has an effect on the development and progression of human ovarian carcinogenesis [30]. When Hh ligand binds to Patch, the inhibition of SMO mediated by Patch is relieved and boosts Gli activation. Gli proteins then move into the nucleus and activate the target genes expression. Conversely, SMO activity is suppressed in the absence of Hh, leading to inhibition of Hh signaling pathway [31]. Besides, the components of the pathway including Ptch and Gli also participate in the feedback regulatory loops to regulate the pathway activity [32]. So far, many downstream target genes of Hh signaling pathway have been found in different-function types, such as cell cycle progression (Cyclin D1 and Cyclin D2), cell fate determination (FOXA2, FOXC2, FOXE1, FOXF1, FOXL1, FOXP3, POU3F1, RUNX2, SOX13, and TBX2), and tumor metastasis (N-Myc and Bcl2) [33]. In this study, overexpression of miR-138 contributes to the loss of SMO mRNA and protein expression and directly targeting its 3'UTR untranslated region. This results suggests that miR-138 can bind to the SMO 3'UTR, thus causing degradation of SMO mRNA, although miRNA functions in both control of translation and mRNA degradation. Hence, the Hh/Ptch/SMO/Gli signaling pathway which usually reactive in HCC patients was blocked due to downregulation of SMO in miR-138-overexpressed HCC cells. Furthermore, the cell growth and invasion of HCC cells were inhibited by miR-138 overexpression or SMO silencing. In addition, previous study showed that miR-138 can induce HCC cells arrest at G1/S phase via targeting Cyclin D3 [16]. Taken together, these findings strongly suggest that miR-138 inhibited HCC cells proliferation, colony formation, invasion and migration partially through inactivation of SMO-mediated Hedgehog signaling pathway, and other target genes of miR-138 may also contribute to the phenomenon, such as Cyclin D3. The feedback loop with components of the Hh pathway may accelerate the destruction of HCC cells. However, the potential Gli target factors responsible for the inhibition of growth and metastasis of miR-138 overexpressed HCC cells are unknown and needs further investigation.

In summary, we found that overexpression of miR-138, as a down-regulated gene in HCC, impaired proliferation and invasiveness of HCC may not all but at least partially though inactivation of SMO-mediated Hh signaling pathway. These results indicate that miR-138 and its relationship with SMO in HCC will provides novel molecular targets for therapeutic strategy.

Disclosure of conflict of interest

None.

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