

Original Article

Upregulation of miR-18 controls the progression of hepatocellular carcinoma by modulating TGF-beta signaling

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Abstract: Aberrant expression of miR-18 was widely reported in various cancers, including prostate cancer and colon cancer. However, its specific role in hepatocellular carcinoma has never been elucidated. The relative level of miR-18 was determined in HCC tissues and cells using real time PCR. The effect of miR-18 on EMT was analyzed by in vitro scratch assays. The changes of EMT markers were determined using western blot analysis. The possible target gene of miR-18 was studied with dual-luciferase reporter assay. Compared with normal para-carcinoma tissue, the level of miR-18 was significantly upregulated in HCC tissues. Meanwhile, compared with LO2 cells, the level of miR-18 was decreased in HepG2, Hep3B, and Sk-Hep1 cells. Western blot analysis showed that transfection with miR-18 inhibitor significantly decreased the expression of E-cad and enhanced the protein expression of α -SMA, fibronectin and vimentin. Moreover, inhibition of miR-18 enhanced cell migration at 24 h and 48 h, respectively. Dual-luciferase assay demonstrated that miR-18 significantly reduced the relative luciferase activity of pmirGLO-Smad2-3'UTR. To conclude, we showed that reduced miR-18 level enhanced HepG2 cell migration and EMT process mainly by targeting Smad2.

Keywords: miR-18, hepatocellular carcinoma, TGF-beta signaling, EMT

Introduction

Hepatocellular carcinoma (HCC) is the most common cancer and the third leading cause of cancer-related mortality worldwide [1, 2]. According to statistics, the global burden of HCC is continuing to rise and it is estimated to be the second highest increase in cancer-related death by 2030 [3]. During hepatocarcinogenesis, a high rate of recurrence after surgery and strong metastasis lead to the extremely poor prognosis of HCC [4]. Therefore, it is of great importance to improve the prognosis of HCC.

Transforming growth factor beta (TGF β) plays a key role in regulating epithelial cell morphology, autonomous tumor initiation, progression and metastasis [5-7]. There are three different isoforms for TGF- β family, including TGF- β 1, TGF- β 2 and TGF- β 3. In the livers, TGF β 1 is the major

form and it exerts its function mainly through Smad proteins [8]. Accompanied by the loss of epithelial markers and the acquisition of migration markers, TGF β 1 enhances the process of epithelial-mesenchymal transition (EMT) [9, 10]. Activation of TGF β 1 signaling leads to the translocation of Smad2 and Smad3 into the nucleus, thereby modulating metastatic process [11]. Therefore, exploring the underlying mechanisms that regulate TGF- β signaling in the metastatic process may shed light on the novel targets for therapeutic interventions in HCC.

MicroRNAs (miRNAs) are small non-coding RNAs that act as important posttranscriptional regulators of gene expression [12]. Previous studies have demonstrated that miRNAs widely participate in the regulation of cell proliferation, differentiation and apoptosis [12-14]. Recently, it was reported that miR-323-3p prompts pan-

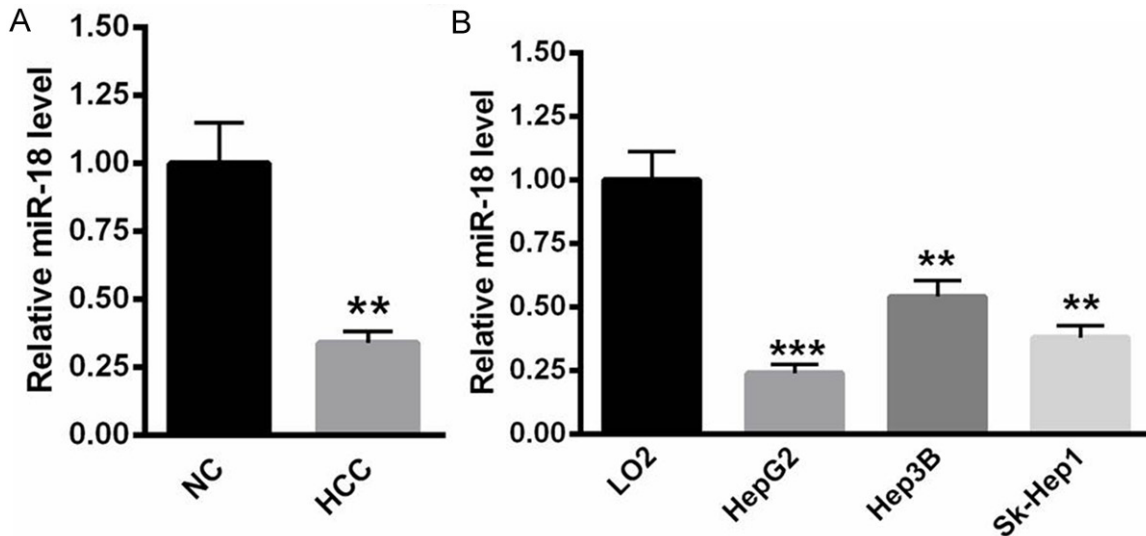


Figure 1. Decreased miR-18 expression in HCC tissues and cell lines. A. Compared with normal para-carcinoma tissue, the level of miR-18 was significantly upregulated in HCC tissues. B. Compared with LO2 cells, the level of miR-18 was increased in HepG2, Hep3B, and Sk-Hep1 cells. Data were presented as mean \pm SE. $n=5$, * $P < 0.05$, ** $P < 0.01$.

creatic ductal adenocarcinoma cell invasion and metastasis mainly by targeting Smad2 and Smad3 [15]. Moreover, enhanced miR-425 expression was reported to enhance esophageal squamous cell carcinoma mainly by binding the 3'untranslated region (3'UTR) of Smad2. Aberrant expression of miR-18 was widely reported in various cancers, including prostate cancer and colon cancer [16, 17]. However, its specific role in hepatocellular carcinoma has never been elucidated.

In the present study, we first demonstrated that the expression level of miR-18 was significantly reduced in the tissues of HCC patients. Furthermore, through suppression the expression of Smad2, decreased miR-18 expression significantly led to the activation of TGF- β signaling, thereby enhancing the metastasis of HCC.

Materials and methods

Cell culture

The human HCC cell lines, HepG2 (well differentiated, low metastatic potential), Hep3B (well differentiated, low metastatic potential), and Human liver adenocarcinoma Endothelial cell line, Sk-Hep1 were purchased from the American Type Culture Collection (Manassas, VA, USA). The normal liver cell line LO2 were ob-

tained from the Committee of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All cell lines were obtained from American Type Culture Collection (Manassas, VA) and cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) (v/v), streptomycin (100 mg/ml) and penicillin (100 IU/ml) at 37°C in a humidified atmosphere containing 5% CO₂.

Transient transfection

Cells were seeded at 10⁶ cells/well in the 6-well plates. Meanwhile, miR-18 mimic, inhibitor, or miR negative control (Genepharma) were mixed with HiperFect transfection reagent (QIAGEN) and incubated at room temperature for 10 min. Then, the complex was added in to the culture medium for 48 h.

RNA extraction and real-time PCR

The total RNA from cultured cells was isolated with TriZol (Invitrogen) according to the manufacturer's instructions. The total RNA was reverse transcribed into complementary DNA (cDNA) with TaqMan RNA Reverse Transcription Kit (Applied Biosystems). A quantitative real-time PCR assay was performed using SYBR Green Supermix (Bio-Rad) in a BIO-RAD iCycler-iQ real-time PCR detection system as previously described [18].

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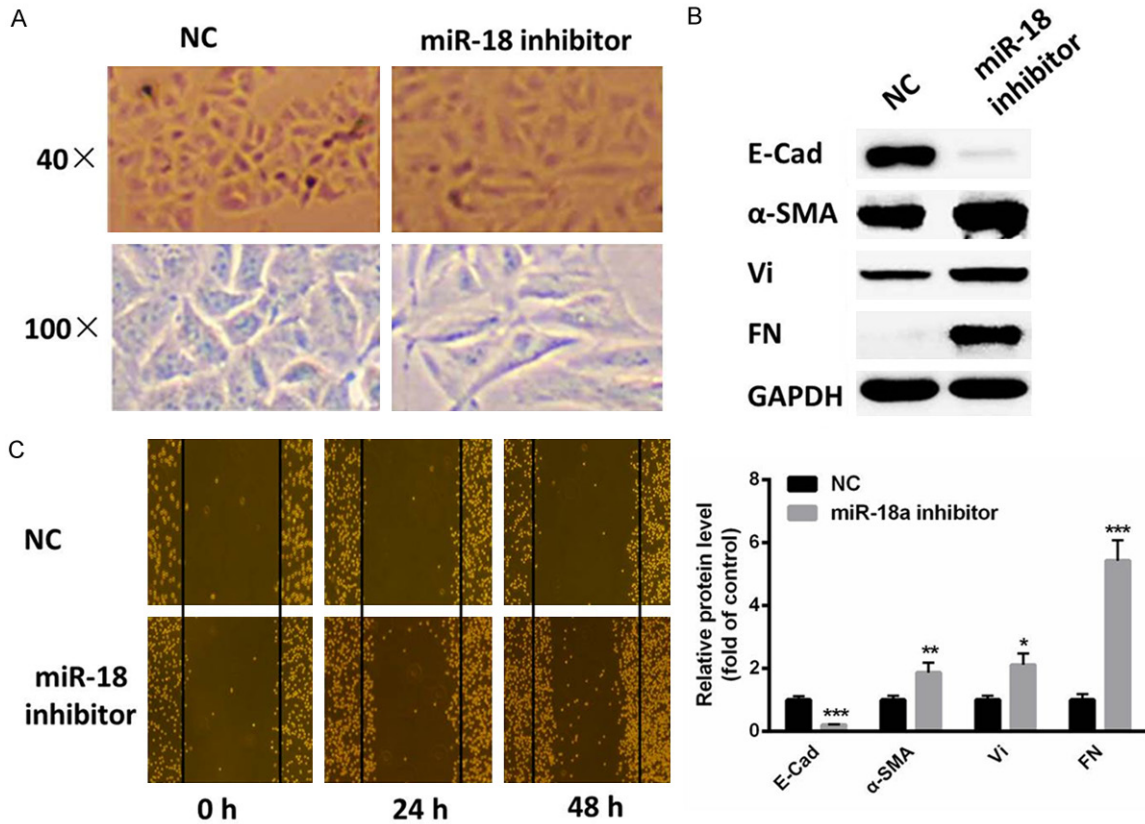


Figure 2. Inhibition of miR-18 prompts the process of EMT and cell migration. A. The cells demonstrated an elongated and spindle-shaped morphology after transfection with miR-18 inhibitor for 48 h. B. Western blot analysis showed that transfection with miR-18 inhibitor significantly decreased the expression of E-cad and enhanced the protein expression of α-SMA, Fibronectin and Vimentin. C. Inhibition of miR-18 enhanced cell migration at 24 h and 48 h, respectively. Data were presented as mean ± SE. n=3, *P < 0.05, **P < 0.01, ***P < 0.001.

Protein extraction and Western blot analysis

Proteins samples were extracted in RIPA buffer (1% TritonX-100, 15 mmol/L NaCl, 5 mmol/L EDTA, and 10 mmol/L Tris-HCl (pH 7.0) (Solarbio, China) supplemented with a protease and phosphatase inhibitor cocktail (Sigma) and then separated by 10% SDS-PAGE, followed by electrophoretical transfer to a PVDF membrane. After soaking with 8% milk in PBST (pH 7.5) for 2 h at room temperature, the membranes were incubated with the following primary antibodies: anti-Smad2, anti-E-cadherin (E-cad), α-SMA, Fibronectin (FN), Vimentin (Vi) and anti-GAPDH (Cell signaling). Immunodetection was performed by enhanced chemiluminescence detection system (Millipore) according to the manufacturer's instructions. The house-keeping gene GAPDH was used as the internal control.

Luciferase target assay

The 3'untranslated region (UTR) of Smad2 containing the predicted binding site was cloned into the pmirGLO (Promega) luciferase reporter vector. The PCR procedures are as follows: a hot start step at 95°C for 10 min, 40 cycles at 95°C for 15 s and 55°C for 45 s, 72°C for 30 s. To construct the mutant vector, the Fast Mutagenesis System was applied (TransGen Biotech, Beijing, China).

For luciferase reporter assay, cells were seeded at 5×10⁴ cells/well in 24-well plates in a 500 μl for 18 h. Then, the modified firefly luciferase vector (500 ng/μl) was mixed with Vigofect transfection reagent according to the manufacturer's instruction. After transfection for 48 h, the Dual-luciferase reporter assay system (Promega) was applied to determine the changes of relative luciferase units (RLU).

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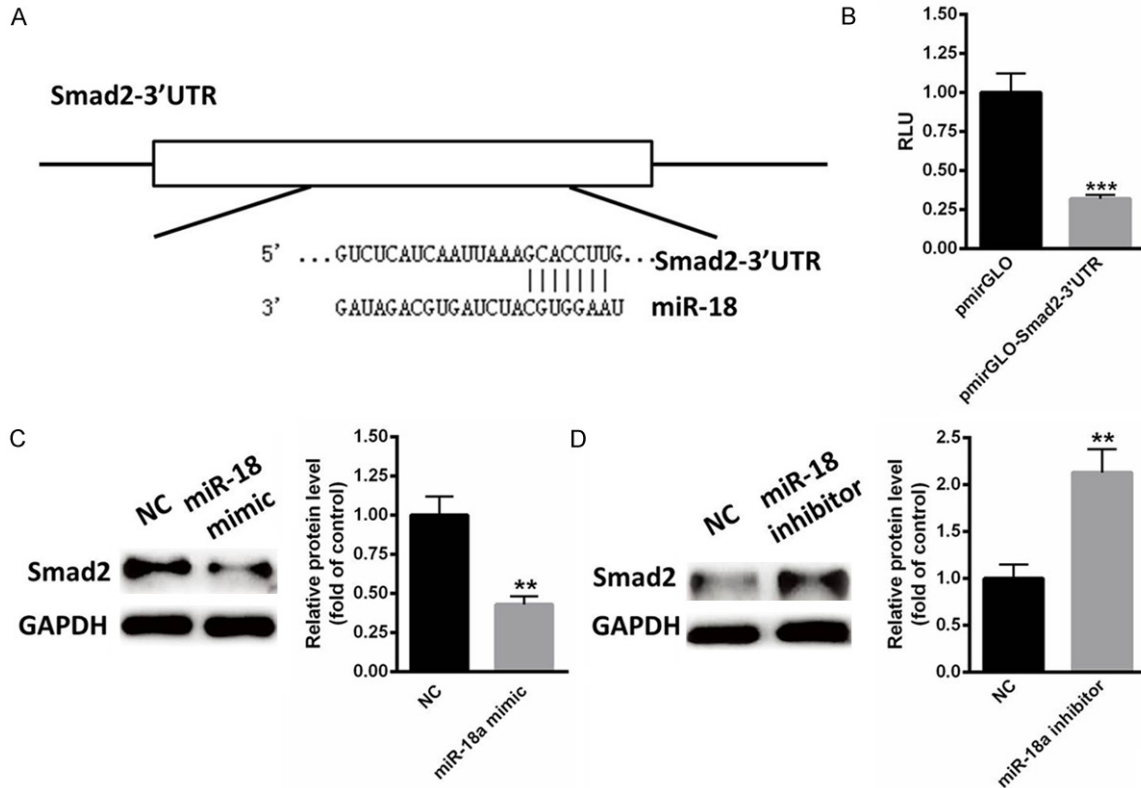


Figure 3. Smad2 is a target gene of miR-18. A. Schematic analysis showed the binding sites of miR-18 in the 3'UTR of Smad2. B. Dual-luciferase assay demonstrated that miR-18 significantly reduced the relative luciferase activity of pmiR-GLO-Smad2-3'UTR. C. Overexpression of miR-18 markedly decreased the protein level of Smad2. D. Inhibition of miR-18 significantly enhanced the expression of Smad2. Data were presented as mean \pm SE. $n=3$, * $P < 0.05$, ** $P < 0.01$.

Renilla activity was used as the internal control.

Migration assay

Cell migration was identified using in vitro scratch assays. Firstly, cells were cultured at 10^5 cells/well in 12-well plates for 24 h. Then, a pipette tip was applied to create an artificial gap in the confluent cell monolayer. After transfection with miR-18 mimic, inhibitor or NC for 48 h, the cells were washed with pre-warmed PBS for three times to remove the debris. The initial images of the scratch (0 h) and final images of the scratch (48 h) were taken.

Statistical analysis

Data were presented as mean \pm SD from 3 independent experiments or 5 mice. Statistical analysis was carried out with Student's t test. $P < 0.05$ was considered as statistically significant difference.

Results

Decreased miR-18 expression in HCC tissues and cell lines

Firstly, we determined the level of miR-18 in HCC tissues. Compared with normal para-carcinoma tissue, the level of miR-18 was significantly upregulated in HCC tissues (**Figure 1A**). Meanwhile, compared with LO2 cells, the level of miR-18 was increased in HepG2, Hep3B, and Sk-Hep1 cells (**Figure 1A**).

Inhibition of miR-18 prompts the process of EMT and cell migration

Then, we explored the possible effects of miR-18 inhibitor on hepG2 cell morphological changes and the expression of EMT markers. As shown in **Figure 2A**, the cells demonstrated an elongated and spindle-shaped morphology after transfection with miR-18 inhibitor for 48 h. Western blot analysis showed that transfection with miR-18 inhibitor significantly de-

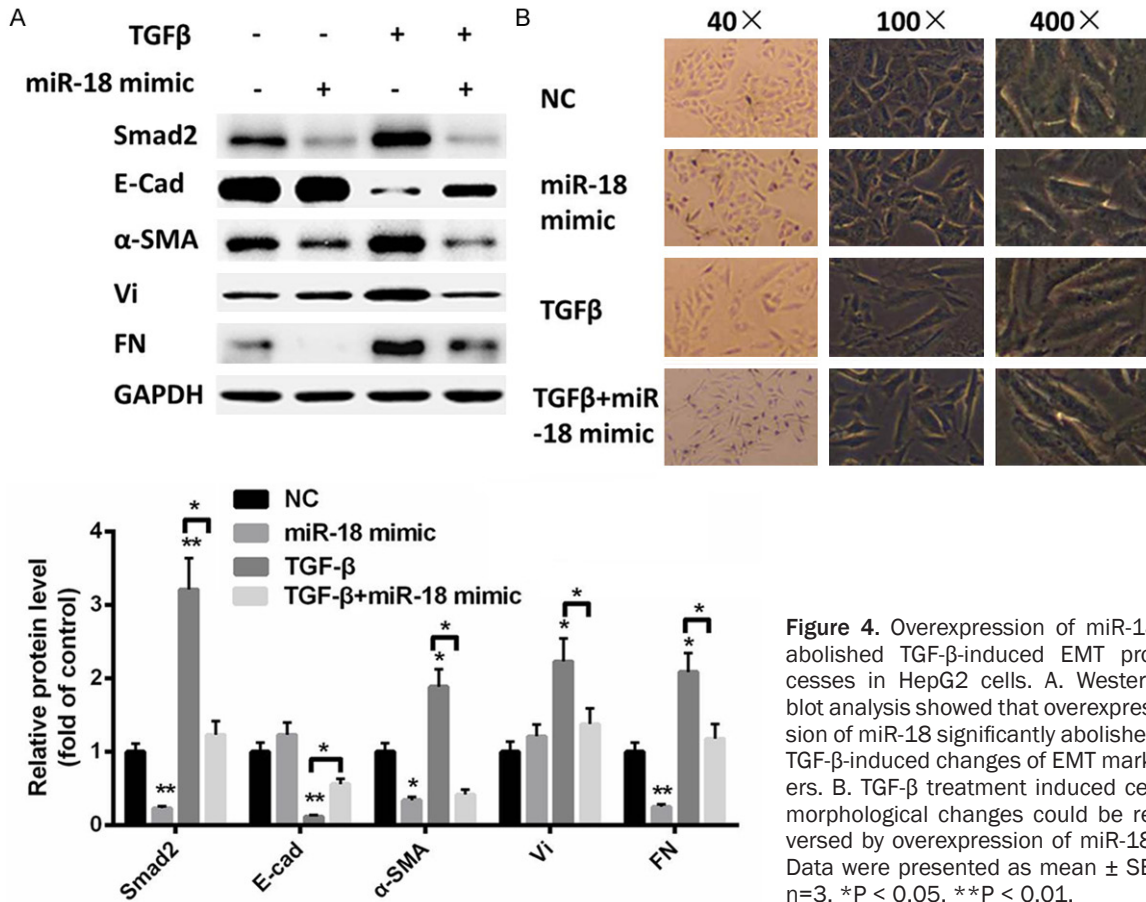


Figure 4. Overexpression of miR-18 abolished TGF-β-induced EMT processes in HepG2 cells. A. Western blot analysis showed that overexpression of miR-18 significantly abolished TGF-β-induced changes of EMT markers. B. TGF-β treatment induced cell morphological changes could be reversed by overexpression of miR-18. Data were presented as mean ± SE. n=3, *P < 0.05, **P < 0.01.

creased the epithelia marker, E-cad. In contrast, inhibition of miR-18 markedly enhanced the protein expression of α-SMA, Fibronectin and Vimentin (Figure 2B). Moreover, we explored the possible role of miR-18 inhibitor on HepG2 cell migration. It was found that inhibition of miR-18 enhanced cell migration at 24 h and 48 h, respectively (Figure 2C). These data suggested that reduced miR-18 level was a key regulator in HepG2 cell morphology changes and migration.

Smad2 is a target gene of miR-18

We then explored the possible target genes of miR-18 that were related to TGF-β. Through TargetScan prediction, a conserved binding site of miR-18 on the 3'UTR of Smad2 was identified (Figure 3A). Dual-luciferase assay demonstrated that miR-18 significantly reduced the relative luciferase activity of pmirGLO-Smad2-3'UTR (Figure 3A). Then, miR-18 mimic or inhibitor was transfected in HepG2 cells for 48 h, respectively. As shown in Figure 3B, overex-

pression of miR-18 markedly decreased the protein level of Smad2. In contrast, inhibition of miR-18 significantly enhanced the expression of Smad2 (Figure 3C). These data suggested that Smad2 was a target gene of miR-18.

Overexpression of miR-18 abolished TGF-β-induced EMT processes in HepG2 cells

Then we try to explore whether overexpression of miR-18 could reverse TGF-β-induced EMT processes in HepG2 cells. As shown in Figure 4A, overexpression of miR-18 significantly decreased the expression of Smad2. Meanwhile, the epithelial marker of E-cad was upregulated, while the protein expression of α-SMA, FN and Vi was reduced. In comparison, TGF-β treatment enhanced the expression of Smad2, accompanied by decreased E-cad expression and enhanced α-SMA, FN and Vi expression. More importantly, we found that overexpression of miR-18 significantly abolished TGF-β-induced changes of EMT markers (Figure 4A). Furthermore, TGF-β treatment induced cell

morphological changes could be reversed by overexpression of miR-18 (Figure 4B).

Discussion

Accumulating evidence has suggested the important role of miRNAs in the progression of HCC. Several miRNAs have been reported to be differentially expressed in HCC tissues, including miR-122, miR-129-2, miR-34 [19-21]. In the present study, we first showed that the level of miR-18 was significantly decreased in both HCC tissues and cell lines. We have shown that inhibition of miR-18 enhanced the process of EMT and cell migration. However, the specific underlying mechanism of miR-18 on HCC progression has never been explored.

TGF- β plays a key role in tumorigenesis mainly through facilitating epithelial to mesenchymal transition by inhibiting E-cadherin [22]. The EMT process refers to the loss of cell-cell contact and the acquisition of fibroblasts properties, inducing cell migration [1, 23]. Then, we explored the effect of miR-18 inhibition on the process of EMT. Interestingly, we found that inhibition of miR-18 markedly enhanced HepG2 cell morphology changes and EMT transitions. Previous study has shown that TGF- β enhances the migration of human HCC cell line, SMMC-7721 cell, through inducing epithelial to mesenchymal transition [24]. Thus, our data showed the possible tumor suppressor role of miR-18 in HCC progression.

Then, we tried to explore the possible target genes of miR-18 that correlate with TGF- β signaling. Dual luciferase reporter assay demonstrated that Smad2 was a target gene of miR-18. Through binding the 3'UTR of Smad2, reduced miR-18 expression enhanced EMT processes in HepG2 cells. Loss of E-Cad and acquisition of the mesenchymal phenotype are common features of EMT [2, 10, 25]. Here, the epithelial marker, E-Cad, and the mesenchymal markers, α -SMA, FN and Vi, were selected in the present study. Here, we demonstrated that overexpression of miR-18 significantly abolished TGF- β -induced upregulation of α -SMA, FN and Vi. Smad-dependent signaling pathway plays a key role in cancer progression. Here, we demonstrated that inhibition of miR-18 prompted cancer cell migration through enhancing EMT process.

The TGF- β -SMAD pathway enhances HCC progression by controlling different stages in the process of cancer cell metastasis, including epithelial-to-mesenchymal transition (EMT) [22, 23]. Abnormal EMT transformation enhances tumor cells migration from the primary site into circulation [26, 27]. Several key regulators are implicated in the process of EMT, including Smad2, Snail, Slug, ZEB2 and FOXC2 [10, 11, 28]. Previous study has shown that overactivation of TGF- β -SMAD2 signaling suppresses the expression of E-cadherin via controlling DNA methylation [11, 27]. Furthermore, enhanced TGF- β expression was reported to induce vessel invasion, liver metastasis, advanced tumor stages and shorter survival times in patient with HCC [29, 30]. In contrast, suppression of TGF- β signaling was effective for the suppression of cell migration in mouse model [31]. Here, we showed that reduced miR-18 level enhanced HepG2 cell migration through increasing EMT process mainly by targeting Smad2.

Here, we first demonstrated that miR-18 plays a key role in the progression of TGF- β signaling, indicating that the miR-18/TGF- β signaling cascade controls the metastatic progression of HCC. Our findings may shed light on novel strategies for the treatment of patients with metastatic HCC.

Disclosure of conflict of interest

None.

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