Downregulation of miR-141 deactivates hepatic stellate cells by targeting the PTEN/AKT/mTOR pathway

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Abstract. The activation of hepatic stellate cells (HSCs) caused by stimulating factors or fibrogenic cytokines is the critical stage of liver fibrosis. Recent studies have demonstrated the influence of microRNAs (miRNAs or miRs) on HSC activation and transformation; however, the function and underlying mechanisms of miRNAs in HSC activation have not yet been completely clarified. In the present study, transforming growth factor β1 (TGF-β1) was used to treat human HSC lines (HSC-T6 and LX2 cells) to simulate the activation of HSCs *in vivo* and whether the expression of miRNAs in HSCs was affected by TGF-β1 treatment was examined using a miRNA microarray. It was observed that miR-141 was one of the most upregulated miRNAs during HSC activation. Functional analyses revealed that miR-141 knockdown suppressed the viability of HSCs and inhibited the expression levels of pro-fibrotic markers. In addition, phosphatase and tensin homolog (PTEN), a well-known suppressor of the AKT/mammalian target of rapamycin (mTOR) pathway, was found to be directly targeted by miR-141 in HSCs. More importantly, the knockdown of PTEN markedly reversed the suppressive effects of miR-141 inhibition on the viability of and the expression levels of pro-fibrotic markers during HSC activation. Finally, it was observed that the downregulation of miR-141 blocked the TGF-β1-induced activation of the AKT/mTOR pathway in HSCs. On the whole, the findings of the present study indicate that miR-141 inhibition suppresses HSC activation via the AKT/mTOR pathway by targeting PTEN, highlighting that miR-141 may serve as a novel therapeutic target for liver fibrosis.

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Introduction

Liver fibrosis is a chronic wound-healing process, which is caused by various damaging factors, including hepatitis B/C, alcoholic liver and non-alcoholic fatty liver diseases (1). Although substantial efforts to clarify the pathogenesis of liver fibrosis have been made, effective therapeutic strategies for this disease are still lacking in clinical practice (2). The activation of hepatic stellate cells (HSCs) is a key step in liver fibrosis, which transform into myofibroblast-like cells upon stimulation by a multitude of signals with the excessive production of extracellular matrix (ECM) components (3,4). Therefore, therapeutic strategies that effectively suppress the activation of HSCs are urgently required.

MicroRNAs (miRNAs or miRs) are a family of short, small, non-coding RNAs which are 21-23 nt in length, and which suppress target gene expression through either translation repression or RNA degradation (5). Increasing evidence has demonstrated that miRNAs play a pivotal role in various physiological and pathological processes, including liver fibrosis (6,7). For example, Wei *et al* reported that miR-455-3p upregulation improved liver fibrosis in mice by suppressing heat shock factor 1 (HSF1) expression (8). Zou *et al* demonstrated that the overexpression of miR-146a attenuated fibrogenesis in a rat model of carbon tetrachloride (CCl4)-induced liver fibrosis (9). Notably, recent studies have revealed that several miRNAs regulate the activation and proliferation of HSCs, which is essential for the pathogenesis of liver fibrosis (10,11). For example, miR-9 has been shown to suppress CCl4-induced liver fibrosis via the regulation of matrix gene expression in HSCs (12). Ju *et al* found that miR-193a/b-3p overexpression inhibited HSC activation and proliferation, which finally resulted in the alleviation of liver fibrosis (13). Therefore, it appears that miRNAs play important roles in the regulation of HSC activation.

In the present study, the miRNA expression profiles during the activation of HSCs were investigated. Subsequently, the role of miR-141 in the activation of HSCs and the regulatory mechanisms were investigated. The findings of the present study provide valuable insight into the complex coordinated regulation of the transforming growth factor (TGF)-β1-induced activation of HSCs by miR-141, which may lead to novel therapeutic strategies for liver fibrosis.

Materials and methods

Tissue samples. Fibrotic liver tissues were obtained from 20 patients with cirrhosis (10 males; mean age, 51.6 years; range, 44-58 years; 10 females; mean age, 53.6 years; range, 49-58 years) at the First Affiliated Hospital of Xinxiang Medical University from January, 2017 to January, 2018. Control liver tissues were obtained from 20 patients with hepatic hemangioma. All experimental protocols were approved by the Ethics Committee of the First Affiliated Hospital of Xinxiang Medical University. Informed consent was obtained from all patients.

Cells and cell culture. The hepatic stellate cell lines, HSC-T6 and LX-2, were obtained from ATCC and maintained in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (HyClone; GE Healthcare Life Sciences) at 37˚C in the presence of 95% air and 5% CO_2. For HSC activation, TGF-β1 (10 ng/ml, cat no. T7039; Sigma-Aldrich; Merck KGaA) was added to the HSC-T6 and LX-2 cells for 0, 6, 12 and 24 h in serum-free DMEM.

miRNA microarray. LX-2 cells were treated with 10 ng/ml TGF-β1 for 0 and 24 h, and total RNA was then extracted using the miRNeasy isolation kit (QIAGEN Milan) according to the manufacturer's instructions. The samples were assessed using the miRCURY LNA™ Array v. 16.0 (Agilent). The procedure and imaging processes were as previously described (14).

RT-qPCR. Total RNA was obtained from liver tissues and cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). miR-141 and mRNA were reverse transcribed using the miScript II RT kit (Qiagen GmbH) and the reverse transcription kit (Invitrogen; Thermo Fisher Scientific, Inc.), respectively. miR-141 and mRNA expression levels were measured using the Exiqon SYBR-Green Master Mix (Exiqon) on a Light Cycler instrument (Bio-Rad Laboratories, Inc.). The PCR cycling conditions were as follows: 5 min at 95˚C, and 36 cycles of 10 sec at 95˚C, 10 sec at 58˚C and 20 sec at 72˚C. The primers used for were as follows: miR-141 forward, 5'-GGGCAT CTTCCAGTACAGT-3' and reverse, 5'-CAGTGCGTGTCG TGGAGT-3'; U6 forward, 5'-TGCGGGTGCTCGCTTCGC AGC-3' and reverse, 5'-CCAGTGCAGGGTCCGAGGT-3'; phosphatase and tensin homolog (PTEN) forward, 5'-GCC CAGACTGCATACGATTT-3' and reverse, 5'-TTGAAGACA CCAAATTTCTGGA-3'; α-smooth muscle actin (α-SMA) forward, 5'-GTTCCAGCCATCCTTCATCGG-3' and reverse, 5'-CCTTCTGCATTCGGTCGGCAA-3'; collagen, type I, α 1 (COL1α1) forward, 5'-ACGGCTCAGAGTCACCCA-3' and reverse, 5'-CCTCCGGTTGATTTCTCATCATA-3'; fibronectin (FN) forward, 5'-GATGCCGATCAGAAGTTTGG-3' and reverse, 5'-GGTTGTGCAGATCTCCTCGT-3'; GAPDH forward, 5'-AGGTCGGTGTGAACGGATTTG-3' and reverse, 5'-TGTAGACCATGTAGTTGAGGTCA-3'. The relative expression levels were calculated based on the $2^{-\Delta\Delta Cq}$ method using U6 and GAPDH as the internal reference for miRNA and mRNA (15). All experiments were performed in triplicate.

Transfection. When cells in a 6-well plate grown to approximately 80% confluence, miR-141 mimics (20 nmol/l), mimics negative control (NC), miR-141 inhibitor (20 nmol/l), inhibitor NC (RiBoBio Guangzhou, China), si-PTEN (30 nM) or si-Scramble (Guangzhou RiboBio Co., Ltd.) were transfected into HSC T6 and LX-2 cells at 37˚C for 48 h, using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The sequences were as follows: miR-141 mimics, 5'-UAA CACUGUCUGGUAAAGAUGG-3'; mimics NC, 5'-UUC UCCGAACGUGUCACGUTT-3'; miR-141 inhibitor forward, 5'-CCAUCUUUACCAGACAGUGUUA-3'; inhibitor NC, 5'-ACUACUGAGUGACAGUAGA-3'; si-PTEN, 5'-GAGCGU GCAGAUAAUGACA-3'; si-Scramble siRNA, 5'-UUCUCC GAACGUGUCACGUTT-3'.

Cell viability. Cell viability was determined using a CCK-8 assay (Beyotime Institute of Biotechnology). At the end of transfection, 10 μl CCK-8 reagent were added to each well, and HSCs were cultured for a further 3 h. The absorbance was then detected using a microplate reader at 450 nm (Bio-Tek Instruments, Inc.).

Luciferase reporter assay. miRNA target prediction tools, including TargetScan Release 7.2 (http://www.targetscan.org/vert_72/) and miRanda (http://miranda.org.uk/) were used to search for the putative targets of miR-141. The dual-luciferase reporter assay was performed as previously described (16). LX-2 cells were transfected with miR-141 mimics or inhibitor and the luciferase reporter plasmids using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). At 48 h post-transfection, luciferase activity was detected using the dual luciferase reporter kit (Beyotime Institute of Biotechnology). Firefly luciferase activity was normalized to *Renilla* luciferase activity.

Western blot analysis. Protein was extracted from the cells using RIPA lysis buffer (Beyotime Institute of Biotechnology) supplemented with a protease inhibitor cocktail (Thermo Fisher Scientific, Inc.). Protein concentration was quantified using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Inc.). A total of 40 μg samples were separated by 12% SDS-PAGE (w/v) and transferred onto a PVDF membrane (Merck KGaA). The membrane was blocked with 5% skim milk for 2 h at room temperature, followed by incubation with primary antibodies against α-SMA (1:1,000; cat. no. 19245), PTEN (1:1,000; cat. no. 9188), phosphorylated (p-)AKT (Ser473; 1:1,000; cat. no. 4060), AKT (1:1,000; cat. no. 4685), p-mammalian target of rapamycin (mTOR) (Ser2448; 1:1,000; cat. no. 5536), mTOR (1:1,000; cat. no. 2983) and β-actin (1:1,000; cat. no. 3700) overnight at 4˚C, followed by incubation with horseradish peroxidase-conjugated mouse anti-rabbit IgG secondary antibodies (1:10,000; cat. no. 5127) at room temperature for 2 h. All antibodies were obtained from Cell Signaling Technology, Inc. The protein bands were detected using an enhanced chemiluminescence (ECL) kit (Thermo Fisher Scientific, Inc.). Semi-quantification was performed using ImageJ version 1.46 (National Institues of Health).

Statistical analysis. Statistical analysis was performed using GraphPad Prism (version 5.0, GraphPad Prism, Inc.). Data are presented as the means ± SD. Differences between groups were analyzed using one-way ANOVA or the Student's t-test

followed by Tukey's post hoc test. Spearman's correlation analysis was used to determine the correlation between miR-141 and PTEN expression. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

miR-141 expression is upregulated in activated HSCs and fibrotic liver tissues. As is known, TGF-β1 has been widely used to simulate HSC activation, which is a key event in the process of liver fibrosis (1,17). Following treatment of the HSC-T6 and LX-2 cells with TGF-β1 (10 ng/ml) for different periods of time, the expression levels of the marker of fibrosis, α-SMA, was evaluated at the protein level. As shown in Fig. 1A, α-SMA expression was markedly increased in the activated HSC-T6 and LX-2 cells, and this effect was time-dependent. In addition, the activation of HSCs was further confirmed by the increased mRNA expression levels of key genes associated with the activation of HSCs, including α-SMA, COL1α1 and FN (Fig. 1B).

In order to analyze the miRNA expression profiles following HSC activation, a miRNA microarray assay was performed using the LX-2 cells treated with TGF-β1 for 0 and 24 h. It was found that 26 miRNAs were upregulated and 31 miRNAs were downregulated following the activation of HSCs (Fig. 1C). Among these aberrantly expressed miRNAs, miR-134-3p and miR-455-5p expression levels were decreased, while miR-214-5p expression was increased, which was consistent with the findings of previous studies (8,18,19), indicating the reliability of the microarray. Notably, miR-141 was selected for subsequent investigation due to its highest expression level in activated HSC group. However, whether miR-141 prevents liver fibrosis through the regulation of HSC activation remains to be elucidated.

Subsequently, miR-141 expression was further assessed by RT-qPCR in the activated HSCs (HSC-T6 and LX-2 cells). As shown in Fig. 1D, miR-141 expression was gradually increased in the activated HSCs. The expression of miR-141 was also measured in liver tissues from 20 patients with cirrhosis and 20 patients with hepatic hemangioma. As was expected, miR-141 expression was markedly increased in the fibrotic liver tissues, compared to the tissues of the control (hemangioma) group (Fig. 1E). Thus, these findings indicate that miR-141 may play a crucial role in the development and progression of liver fibrosis.

Knockdown of miR-141 inhibits the TGF-β1-induced activation of HSCs. To determine whether miR-141 affects the activation of HSCs, HSC-T6 and LX-2 cells were transfected with miR-141 inhibitor/mimics, followed by stimulation with 10 ng/ml TGF-β1. As shown in Fig. 2A, miR-141 expression was notably increased following transfection with miR-141 mimics, whereas it was decreased following transfection with miR-141 inhibitor, demonstrating the sufficient transfection efficacy. Abnormal cell proliferation is an obvious and important parameter of HSC activation (20). Thus, the present study examined whether the inhibition of miR-141 affects this parameter. The results of CCK-8 assay revealed that the increased proliferation of LX-2 and HSC-T6 cells induced by TGF-β1 was significantly suppressed by the knockdown of miR-141 (Fig. 2B and C). Additionally, the knockdown of miR-141 significantly decreased the levels of α-SMA in the activated HSC-T6 and LX-2 cells at the protein level (Fig. 2D). Furthermore, the mRNA expression levels of α-SMA, COL1α1 and FN in the activated HSC-T6 and LX-2 cells were also found to be decreased following the knockdown of miR-141 (Fig. 2E and F). All these data suggest that miR-141 inhibition suppresses the activation of HSCs induced by TGF-β1.

PTEN is a target of miR-141 in HSCs. To explore the mechanisms involved in the inhibitory effects of miR-141 on HSC activation, online software, including TargetScan (www.targetscan.org/vert_72/) and miRanda (http://miranda.org.uk/) were used to predict the relevant targets of miR-141. As shown in Fig. 3A, PTEN, a well-known suppressor of the AKT/mTOR pathway, was identified as a potential target of miR-141. In a previous study, it was demonstrated that PTEN was a direct target of miR-141 in nasopharyngeal carcinoma (NPC) (21). However, the association between miR-141 and PTEN in liver fibrosis has not yet been clarified. Thus, the present study first detected the expression of PTEN in fibrotic liver tissues by RT-qPCR and the results revealed that PTEN expression was markedly downregulated in the fibrotic liver tissues, compared to the hemangioma control group (Fig. 3B). Moreover, there was an inverse correlation between PTEN and miR-141 expression levels in the fibrotic liver tissues (Fig. 3C). Subsequently, the effect of miR-141 on the expression of PTEN was measured at the protein level in HSCs by western blot analysis. As shown in Fig. 3D, the protein expression of PTEN was significantly downregulated following transfection with miR-141 mimics in the HSCs. To further examine the association between miR-141 and PTEN, a luciferase assay was conducted. It was observed that overexpression of miR-141 decreased, whereas the knockdown of miR-141 increased the relative luciferase activity of PTEN 3'-UTR wt. However, no significant differences were found in luciferase activity when the LX-2 cells were co-transfected with PTEN 3'-UTR mut reporter and miR-141 mimics/inhibitor (Fig. 3E). In addition, the effect of miR-141 on the expression of PTEN in activated HSCs was examined and the results revealed that the expression level of PTEN was markedly downregulated in the activated HSC-T6 and LX-2 cells; the effects of TGF-β1 on PTEN expression were reversed by transfection with miR-141 inhibitor (Fig. 3F). These results indicated that PTEN may be a functional target of miR-141 during the activation of HSCs.

Knockdown of miR-141 suppresses HSC activation by targeting PTEN. To confirm whether miR-141 mediates the activation of HSCs by targeting PTEN, HSCs were transfected with si-PTEN and miR-141 inhibitor, followed by TGF-β1 treatment. Initially, the transfection efficiency of si-PTEN was evaluated by western blot analysis. It was shown that PTEN was notably down-regulated after si-PTEN transfection in both LX-2 and HSC-T6 cells (Fig. 4A). The results of CCK-8 assay revealed that miR-141 inhibition played a suppressive role in the proliferation of LX-2 and HSC-T6 cells induced by TGF-β1, whereas transfection with si-PTEN partially reversed this inhibitory effect of miR-141 (Fig. 4B and C). Moreover, miR-141 inhibition significantly decreased the levels of α-SMA in the activated HSCs at the protein level,

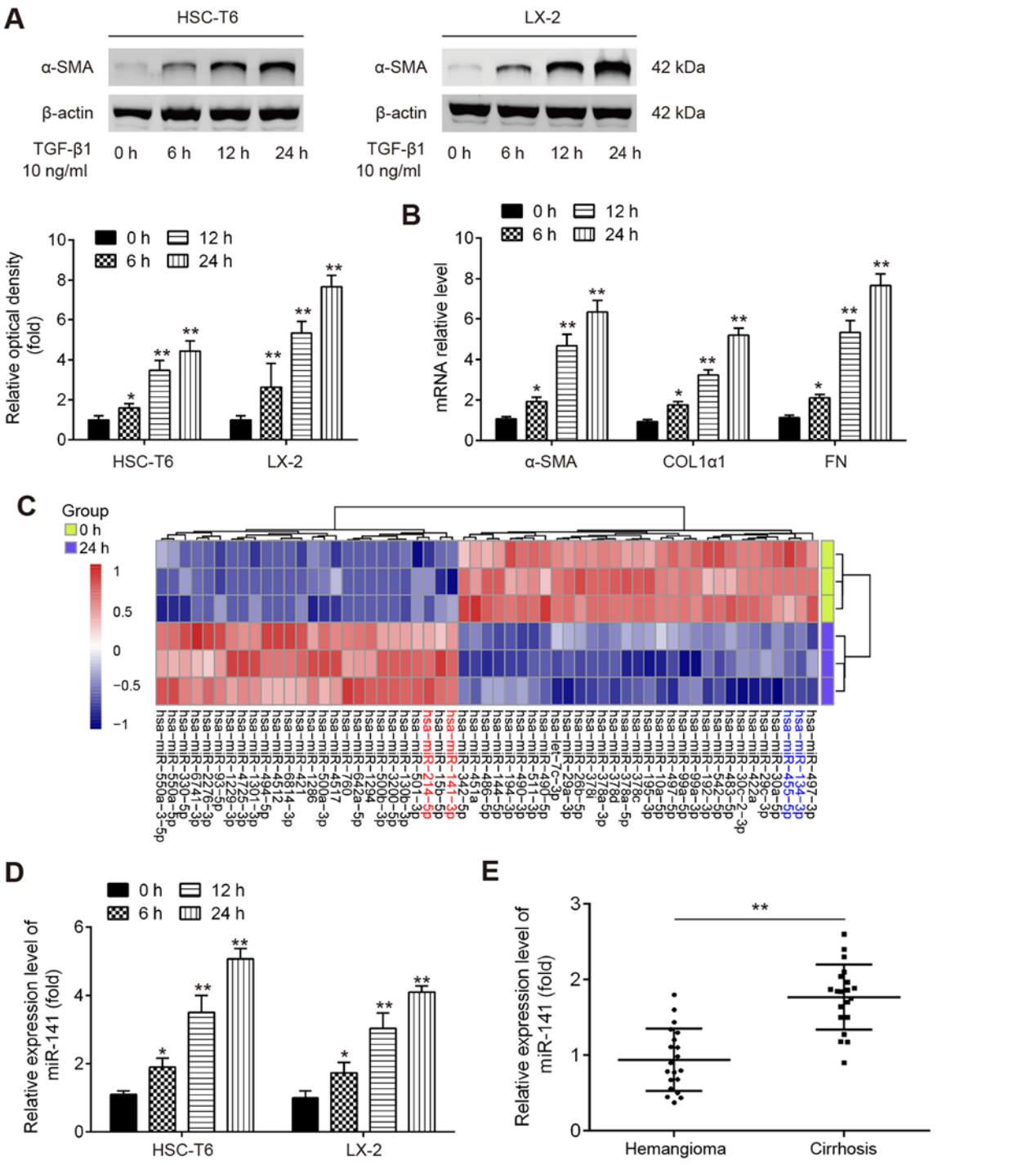

Figure 1. miR-141 is upregulated in fibrotic liver tissues and in activated HSCs induced by TGF-β1. LX-2 and HSC-T6 cells were treated with 10 ng/ml TGF-β1 for 24 h, and the cells were then harvested for subsequent analysis. (A) The protein level of α-SMA was measured in LX-2 and HSC-T6 cells by western blot analysis. Data represent the means ± SD of 3 independent experiments. *P<0.05, **P<0.01 vs. control group. (B) The mRNA levels of α-SMA, COL1α1 and FN were determined by RT-qPCR. Data represent the means ± SD of 3 independent experiments. *P<0.05, **P<0.01 vs. control group. (C) Microarray analysis of miRNA expression was performed using total RNAs extracted from resting and activated HSC-T6 cells. (D) The expression level of miR-141 in TGF-β1 activated LX-2 and HSC-T6 cells was examined by RT-qPCR. Data represent the means ± SD of 3 independent experiments. *P<0.05, **P<0.01 vs. control group. (E) The expression level of miR-141 in fibrotic liver tissues was examined by RT-qPCR (n=20). **P<0.01 vs. control (hemangioma) group. HSCs, of hepatic stellate cells; TGF-β1, transforming growth factor β1.

while this suppressive effect was also reversed by transfection with si-PTEN (Fig. 4D). Similarly, the effects of miR-141 on the mRNA expression levels of α-SMA, COL1α1 and FN were reversed by transfection with si-PTEN (Fig. 4E and F). All these data indicate that the miR-141/PTEN axis plays an important role in regulating HSC activation.

miR-141 suppresses HSC activation through the Akt/mTOR pathway. PTEN is a critical regulator of the Akt/mTOR pathway that can induce fibrogenesis in HSCs (22-24). In the present study, to determine whether the PTEN downstream effector, the Akt/mTOR pathway, was involved in HSC activation, the phosphorylation/activation of the

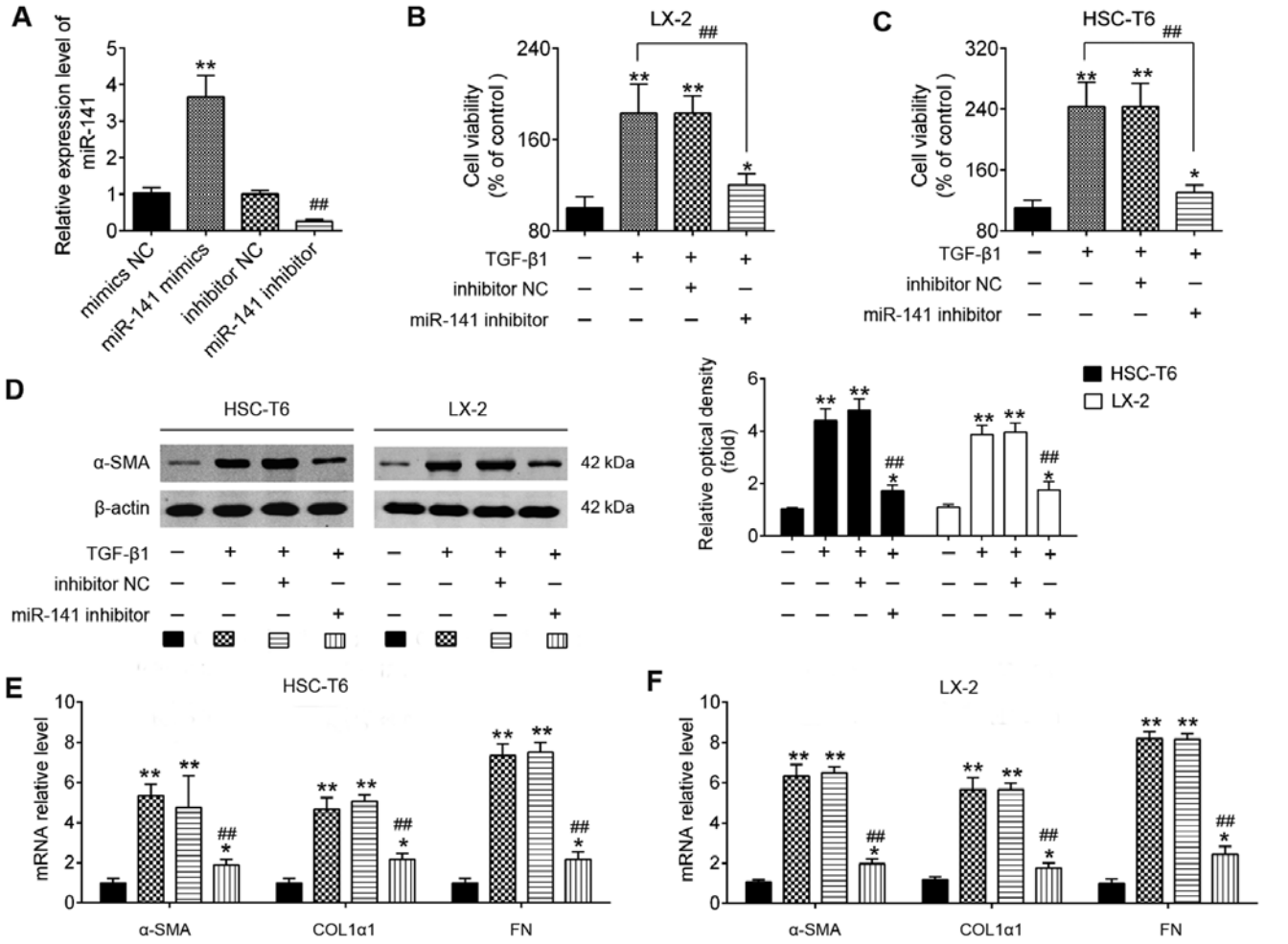

Figure 2. Knockdown of miR-141 inhibits the activation of HSCs induced by TGF-β1. (A) The expression level of miR-141 was examined in LX-2 and HSC-T6 cells following transfection with miR-141 mimics or inhibitor (20 nmol/l). Data represent the means ± SD of 3 independent experiments. **P<0.01 vs. mimics NC group. $^{\#\#}$P<0.01 vs. inhibitor NC group. HSC-T6 and LX-2 cells were transfected with miR-141 inhibitor (20 nmol/l) and after 24 h, the cells were stimulated with 10 ng/ml TGF-β1 for a further 24 h, and the cells were then harvested for use in subsequent experiments. (B and C) Proliferation of LX-2 and HSC-T6 cells was detected by CCK-8 assay. (D) The protein level of α-SMA was measured by western blot analysis. (E and F) The mRNA expression levels of α-SMA, COL1α1 and FN were detected by RT-qPCR in HSC-T6 and LX-2 cells, respectively. Data represent the means ± SD of 3 independent experiments. *P<0.05, **P<0.01 vs. control group. $^{\#\#}$P<0.01 vs. TGF-β1 alone group. HSCs, of hepatic stellate cells; TGF-β1, transforming growth factor β1; α-SMA, α smooth muscle actin; COL1α1, collagen, type I, α 1; FN, fibronectin.

Akt/mTOR signaling pathway was examined by western blot analysis. The results revealed that the expression of PTEN was markedly decreased, and the expression levels of p-Akt and p-mTOR were increased in the TGF-β1-treated HSC-T6 and LX-2 cells, compared to the control group, suggesting that TGF-β1 activates the Akt/mTOR pathway. However, miR-141 inhibition blocked the TGF-β1-induced activation of the Akt/mTOR pathway, as evidenced by the induction of PTEN expression and the reduction of p-AKT and p-mTOR expression (Fig. 5). These data thus suggest that miR-141 may suppress the activation of HSCs by inhibiting the activation of the PTEN/Akt/mTOR pathway.

Discussion

In the present study, miR-141 expression was found to be upregulated in activated HSCs and fibrotic liver tissues. Moreover, it was demonstrated that miR-141 knockdown suppressed the activation of HSCs by inhibiting the activation of the PTEN/Akt/mTOR pathway. These findings indicate that the downregulation of miR-141 expression may provide a novel therapeutic strategy for liver fibrosis.

A large body of evidence indicates that miRNAs have emerged as important layers of regulatory control from quiescent HSCs into activated myofibroblast-like cells (activated HSCs) (25-27). For example, Li *et al* demonstrated that miR-27a/b-3p reduced the increased α-SMA expression that can directly increase the contractility of fibroblasts by incorporating into stress fibers and α1 (I) collagen levels in cultured HSCs (28). Wang *et al* found that miR-454 inhibited the activation and proliferation of HSCs by suppressing the expression of Wnt10a, to reduce liver cirrhosis (29). Sekiya *et al* reported that miR-29b expression was decreased in activated HSCs, and that miR-29b upregulation suppressed HSC activation (30). Similarly, Wang *et al* found that miR-29b repressed HSC activation by inhibiting the PI3K/AKT pathway (31). Ji *et al* demonstrated that the knockdown of miR-27a/b maintained HSCs in a quiescent state and decreased the cell proliferation rate (32). However, some authors have reported that several miRNAs promote HSC activation and liver fibrosis. For

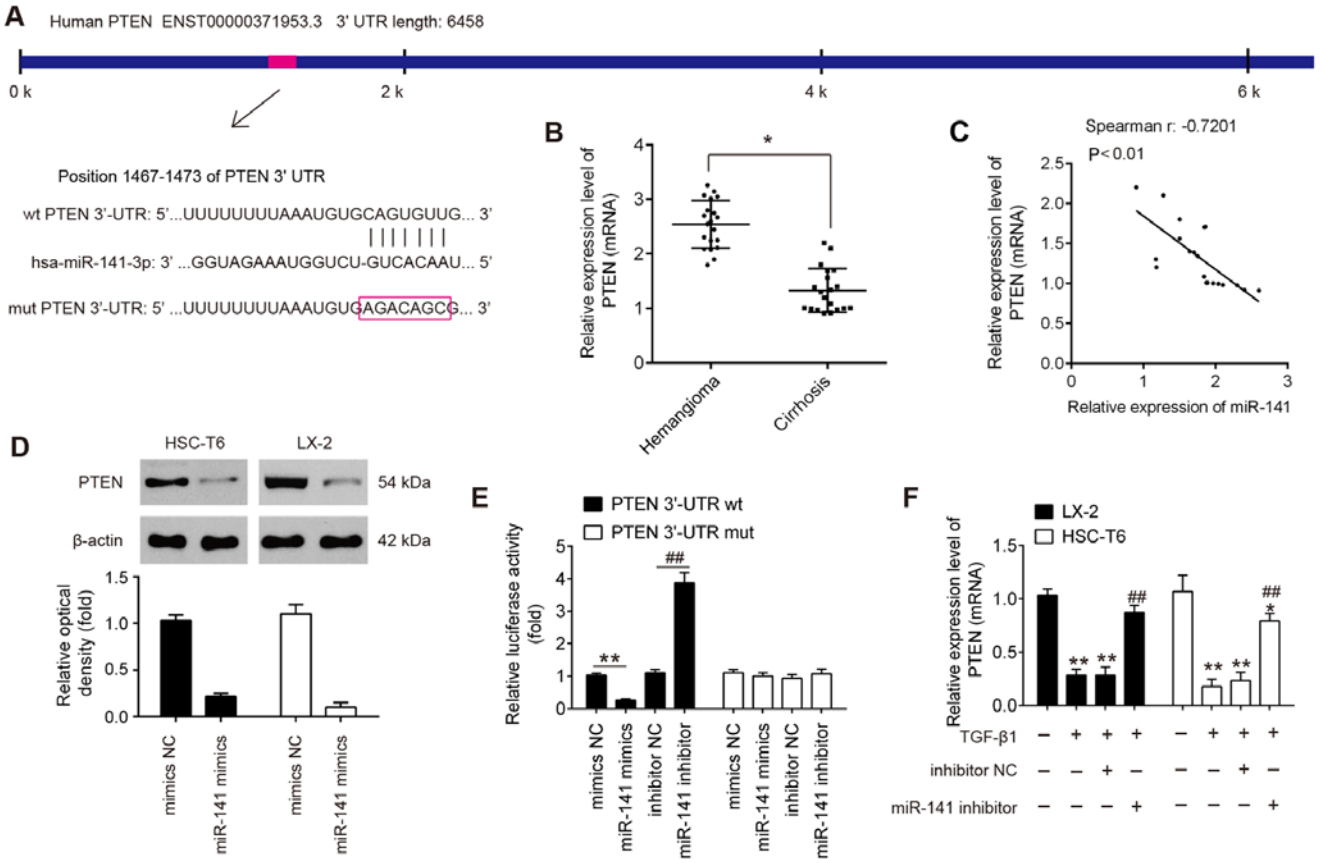

Figure 3. PTEN is a direct target of miR-141 in HSCs. (A) Predicted miR-141 targeting sequence in PTEN 3'UTR (wt PTEN 3'-UTR). Target sequences of PTEN 3'-UTR were mutated (mut PTEN 3'-UTR). (B) The expression level of PTEN in fibrotic liver tissues was examined by RT-qPCR [*P<0.01 vs. control (hemangioma) group]. (C) Spearman's rank correlation was used to analyze the correlation between miR-141 and PTEN expression levels in fibrotic liver tissues (r=-0.7201, P<0.01). (D) The protein levels of PTEN were detected by western blot analysis following transfection of LX-2 and HSC-T6 cells with miR-141 mimics and inhibitor (20 nmol/l). (E) Luciferase assay of LX-2 cells co-transfected with firefly luciferase constructs containing the PTEN wt or mut 3'-UTRs and miR-141 mimics, mimic NC, miR-141 inhibitor or inhibitor NC, as indicated (n=3). **P<0.01 vs. mimics NC group. $^{\#\#}$P<0.01 vs. inhibitor NC group. (F) HSC-T6 and LX-2 cells were transfected with miR-141 inhibitor and after 24 h, the cells were stimulated with 10 ng/ml TGF-β1 for a further 24 h, and the mRNA levels of PTEN were then detected by RT-qPCR. Data represent the means ± SD of 3 independent experiments. *P<0.05, **P<0.01 vs. control group. $^{\#\#}$P<0.01 vs. TGF-β1 group. HSCs, of hepatic stellate cells; TGF-β1, transforming growth factor β1; PTEN, phosphatase and tensin homolog.

example, miR-214 has been shown to promote HSC activation and liver fibrosis by suppressing Sufu expression to modulate the Hedgehog signaling pathway (18). However, the underlying mechanisms through which miRNAs regulate HSC activation remain largely unknown. In the present study, using a miRNA microarray assay, it was found that miR-141 was expression upregulated in TGF-β1-treated HSCs *in vitro*. It was also found that miR-141 expression was upregulated in fibrotic liver tissues from patients with cirrhosis, which was in accordance with the results obtained with activated HSCs. These results suggested that miR-141 may play critical roles in the pathogenesis of liver fibrosis.

Several studies have demonstrated that miR-141 is involved in fibrosis (33,34). For example, Tan *et al* demonstrated that miR-141 expression was increased in serum samples from patients with primary biliary cirrhosis, indicating that the aberrant expression of miR-141 may be closely associated with liver fibrosis (35). Another study reported that miR-141 inhibition alleviated diabetic renal fibrosis in rats with diabetic kidney disease (DKD) (34). However, it remains unclear as to whether miR-141 is involved in HSC activation that results in liver fibrosis. In the present study, HSC-T6 and LX-2 cells were used to investigate the effects of miR-141 on the activation of HSCs. As ws expected, the activated HSCs displayed a significant increase in cell viability and fibrotic marker expression, including α-SMA, COL1α1 and FN. Of note, the inhibition of miR-141 reduced the expression of these fibrotic markers in the culture-activated HSCs. The findings confirmed that miR-141 inhibition may attenuate liver fibrosis by suppressing HSC activation. Notably, apart from miR-141, a number of other miRNAs were also upregulated in the TGF-β1-treated HSCs. Therefore, further investigations are required to reveal whether other miRNAs are involved in the regulation of HSC activation, which may not be limited to targeting miR-141-3p.

PTEN has been shown to play important roles in fibrotic diseases, including the in lungs, heart, skin and liver (36-40). For example, Zheng *et al* reported that the dynamic expression of PTEN in rat liver tissues was negatively associated with liver fibrosis (37). He *et al* found that the loss of PTEN in mice resulted in the excess deposition of type I collagen, while PTEN overexpression reversed chemical-induced liver fibrosis (41). Of note, previous studies have reported that miRNAs play important roles in the regulation of HSC activation by targeting PTEN. For example, Niu *et al* demonstrated that miR-1273g-3p affected the activation of HSCs by directly targeting PTEN in hepatitis C virus (HCV)-related liver fibrosis (42). Zheng *et al* found that

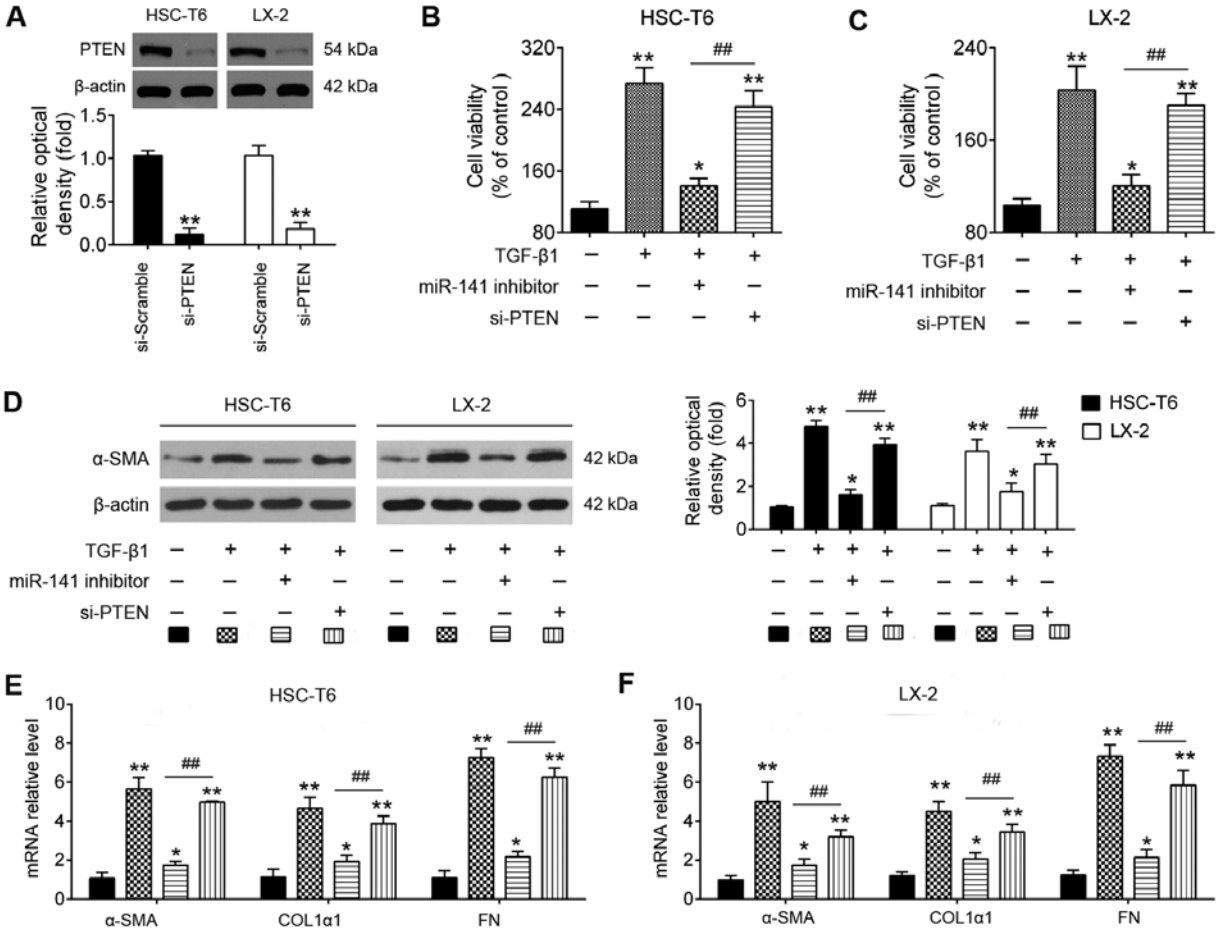

Figure 4. miR-141 mediates HSC activation and proliferation by targeting PTEN. (A) The expression level of PTEN was examined in LX-2 and HSC-T6 cells following transfection with si-PTEN by western blot analysis. The miR-141 inhibitor (20 nmol/l) and si-PTEN (30 nmol) were transfected into the HSC-T6 and LX-2 cells, followed by stimulation with 10 ng/ml TGF-β1 for 24 h, and the cells were then harvested for use in subsequent experiments. (B and C) Proliferation of LX-2 and HSC-T6 cells was detected by CCK-8 assay. (D) The protein level of α-SMA was measured by western blot analysis. (E and F) The mRNA expression levels of α-SMA, COL1α1 and FN were detected by RT-qPCR in HSC-T6 and LX-2 cells, respectively. Data represent the means ± SD of 3 independent experiments. *P<0.05, **P<0.01 vs. control group. $^{\#\#}$P<0.01 vs. miR-141 inhibitor group. HSCs, of hepatic stellate cells; TGF-β1, transforming growth factor β1; PTEN, phosphatase and tensin homolog; α-SMA, α smooth muscle actin; COL1α1, collagen, type I, α 1; FN, fibronectin.

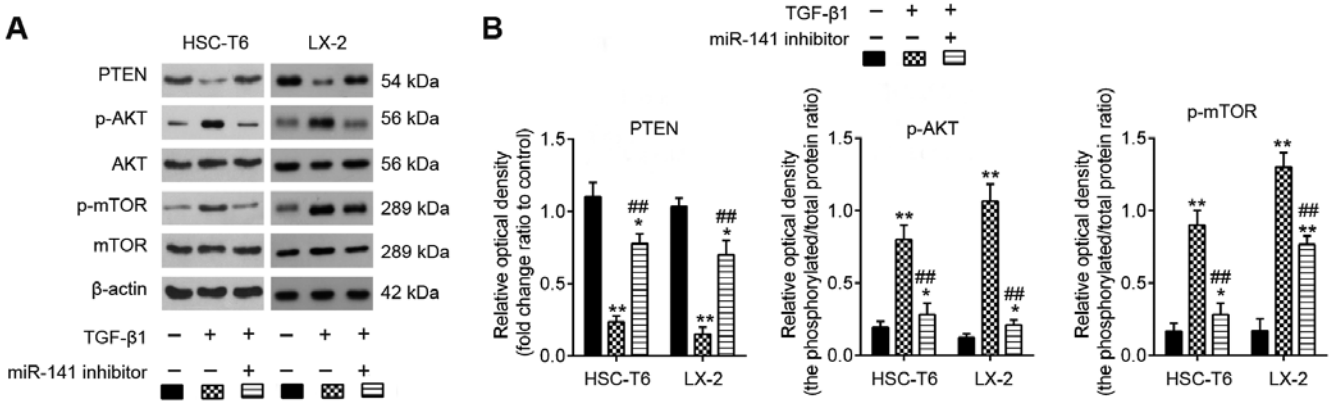

Figure 5. miR-141 blocks the activation of AKT/mTOR pathway via the promotion of PTEN. The miR-141 inhibitor (20 nmol/l) was transfected into the HSC-T6 and LX-2 cells, and after 24 h, the cells were stimulated with 10 ng/ml TGF-β1 for a further 24 h, and the cells were then harvested for use in subsequent experiments. (A) The protein expression levels of PTEN, AKT, p-AKT, mTOR and p-mTOR were determined by western blot analysis. (B) The bands were semi-quantitatively analyzed using ImageJ software, normalized to β-actin or total protein density. Data represent the means ± SD of 3 independent experiments. *P<0.05, **P<0.01 vs. control group; $^{\#\#}$P<0.01 vs. TGF-β1 group. HSCs, of hepatic stellate cells; TGF-β1, transforming growth factor β1; PTEN, phosphatase and tensin homolog; mTOR, mammalian target of rapamycin.

miR-181b overexpression activated HSCs by suppressing PTEN expression (43). In the present study, bioinformatics analysis was performed to predicate the putative targets of miR-141 and PTEN was identified as the potential target of miR-141.

In addition, the expression of PTEN was markedly decreased, and inversely correlated with miR-141 expression in the fibrotic liver tissues. More importantly, the knockdown of PTEN attenuated the inhibitory effects of miR-141 downregulation on HSC activation, suggesting that miR-141 inhibited HSC activation by promoting the expression of PTEN.

It is well known that PTEN negatively regulates the AKT/mTOR pathway, which has been reported to facilitate collagen synthesis in fibroblasts associated with various fibrotic diseases, including liver fibrosis (43-45). For example, Son *et al* demonstrated that the inhibition of the AKT/mTOR pathway suppressed the activation of HSCs (46). Reif *et al* have shown that inhibition of AKT/mTOR pathway by LY294002, an inhibitor of PI3K, attenuated the activation of HSCs in liver fibrosis (47). Several miRNAs have shown important roles in the regulation of HSCs activation via the Akt/mTOR pathway. For example, Wu *et al* found that miR-140 contributed to HSC activation by suppressing the activation of the AKT/mTOR pathway (48). Given the correlation between miR-141 and PTEN, it was thus hypothesized that miR-141 may affect HSC activation by regulating the AKT/mTOR pathway. In the present study, it was observed that TGF-β1 promoted the expression levels of p-Akt and p-mTOR in HSC-T6 and LX-2 cells, whereas the inhibition of miR-141 inhibited these TGF-β1-induced effects. These findings suggest that miR-141 may suppress HSC activation by inhibiting the activation of the PTEN/AKT/mTOR pathway.

In conclusion, the present study demonstrated that miR-141 expression was upregulated in activated HSCs and fibrotic liver tissues, and that miR-141 knockdown suppressed HSC activation by targeting PTEN, which inhibited the activation of the AKT/mTOR pathway. These findings suggest that miR-141 may be a potential therapeutic target for liver fibrosis.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

DY conceived and designed the experiments. HaijunL, XW, CS, YD, BC and HaixiaL performed the experiments. HaijunL, XW, CS, YD, BC and HaixiaL analyzed the data. DY contributed the reagents/materials/analysis tools and wrote the manuscript. All authors have read and agreed to the final version of manuscript.

Ethics approval and consent to participate

All individuals provided informed consent for the use of human specimens for clinical research. The present study was approved by the First Affiliated Hospital of Xinxiang Medical University Ethics Committees.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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