Original Article
MicroRNA-377 is associated with portal hypertension in rat liver cirrhosis by repressing heme oxygenase-1

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Abstract: Heme oxygenase-1 (HO-1) enzyme plays a critical role in the development and progression of portal hypertension liver cirrhosis. In the present study, we hypothesized that a post-translational mechanism of micro-RNAs regulated the expression of HO-1 in a rat model of cirrhosis. MiRNA-377 as a candidate was screened by miRNA microarray. MiRNA-377 was significantly lower in cirrhotic tissue than that of the control group. Moreover, the levels of HO-1 were increased in liver tissues as compared to those of healthy control group. Intriguingly, bioinformatics analysis suggested that miRNA-377 was a regulator of HO-1, and miRNA-377 regulated HO-1 through the predicted binding sites in its 3'-UTR. MiRNA-377 via targeting HO-1 regulated the cell migration in HSCs and LX-2 cell lines. The levels of the profibrotic markers α-SMA and collagen-1 were significantly down-regulated after transfected with miR-377 mimic; in contrast, α-SMA and collagen-1 were significantly up-regulated in the present of CoPP in HSCs and LX-2 cell lines. In vivo, α-SMA and collagen-1 were significantly up-regulated in the cirrhotic portal hyperpietic rats and CoPP treatment cirrhotic rats, in contrast, ZnPP treatment could inhibited α-SMA and collagen-1 expression in cirrhotic portal hyperpiesia rats as compared to that of the PH group. In conclusion, these results suggest that the down-regulation of miRNA-377 may be involved in development and progression of portal hypertension liver cirrhosis.

Keywords: Heme oxygenase-1, portal hypertension liver cirrhosis, microRNA-365, zinc protoporphyrin, cobalt protoporphyrin

Introduction

Portal hypertension is a serious consequence of liver cirrhosis that results in life-threatening complications, such as hyperdynamics splanchnic circulation, an extensive network of porto-systemic collaterals, intestine inflammation and splenomegaly [1], with elevated morbidity and mortality [2]. In cirrhotic livers, impairment of the intrahepatic microcirculation is the result of both structural changes and an increased intrahepatic vascular tone, which is the initial factor for portal hypertension progression and development [3]. Previous studies demonstrate that dysfunction of intrahepatic vascular tone in liver cirrhosis originates from the downregulation of endogenous vasodilators, including glucagon, prostacyclin and nitric oxide [4, 5].

However, the underlying molecular mechanisms in portal hypertension-induced hepatic injury have not been clearly delineated.

MicroRNAs (miRNAs) are endogenous non-coding RNAs and single-stranded RNA molecules of ~22 nucleotides in length that regulate 20-80% of the host genes and serve as important post-transcriptional gene regulators [6, 7]. The key features of miRNAs control cell proliferation and differentiation of various cell types. A growing number of studies have demonstrated that the pathogenic change in various of tissues has been linked to miRNAs [8, 9]. Interestingly, multiple miRNAs are involved in hepatic diseases, such as viral hepatitis, hepatic fibrosis and hepatocellular carcinoma (HCC) [6, 10, 11]. Heme oxygenase-1 (HO-1) is a

32-kDa microsomal/mitochondrial enzyme, which oxidizes protoheme to biliverdin IXα in a three-step process. In the process, HO-1 releases the antioxidant molecules carbon monoxide and biliverdin [12]. Previous studies indicate that HO-1 levels are elevated in in cirrhotic rat liver and regulates intrahepatic vascular tone through carbon monoxide (CO) production [13]. Moreover, HO-1 levels are associated with local hemodynamic changes in patients with chronic portal hypertension, and increased HO-1 gene expression is detected in the livers of patients with portal hypertension due to severe hepatic cirrhosis [5, 14]. These results suggest that HO-1 plays a pathophysiological role in cirrhotic portal hypertension.

In the present study, we hypothesized that a post-translational mechanism might exist for HO-1 expression and be regulated by miRNAs in cirrhotic portal hyperpiesia rats. Our results demonstrated that miRNA-377 regulated HO-1 protein expression through the predicted binding sites in its 3'-UTR.

Materials and methods

Patients' samples

Thirty-one human cirrhotic tissues and eighteen healthy control subjects were collected from the Department of Hepatobiliary Surgery, Affiliated Hospital of Binzhou medical College between Jan 2012 and June 2014. All collected tissue samples were immediately stored at liquid nitrogen until use. Human samples were obtained with written informed consent from all patients. The study was approved by the Ethics Committee of the Binzhou medical College, China.

Cell culture and animal model

Primary hepatic stellate cells (HSCs) and LX-2 cell lines were obtained from the Cell Resource Center, Shanghai Institutes for Biological Sciences (SIBS, China). HSCs and LX-2 were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco Life Technologies) with 1 g/L glucose and 10% FCS at 37°C in a humidified incubator (Thermo, USA), 5% CO_2, 95% air atmosphere.

The rats were randomly divided into four groups: (1) sham operated control rats (sham, n=10); (2) portal hypertension liver cirrhosis rats (PH, n=10); (3) ZnPP treatment group (ZnPP, n=10); (4) CoPP treatment group (CoPP, n=10). Biliary cirrhosis was induced by bile duct ligation (BDL) in rats of the cirrhotic group, ZnPP and CoPP treatment groups. Rats in ZnPP and CoPP treatment groups received an intraperitoneal injection with ZnPP or CoPP 30 mg/kg/day.

Luciferase reporter gene activity assay and migration assay

The 3'UTR of HO-1 gene containing the predicated target sites for miR-377 was obtained by PCR amplification. The fragment was inserted into the multiple cloning sites in the pMIR-REPORT luciferase microRNA expression reporter vector (Ambion, Austin, USA). HEK-293 cells were co-transfected with 0.1 μg of luciferase reporters containing HO-1 3'UTR and miR-377 mimics by Lipofectamine 2000 (Invitrogen, Carlsbad, USA). We harvested the cell lysates after 48 h transfection and measured the luciferase activity with a dual luciferase reporter assay kit according to manufacturer's instruction. Migration assay used Transwell inserts (Costar, NY, USA; 8-mm pore size) in 24-well dishes.

Overexpression of miR-377

For the transfection of HSCs and LX-2, lentiviral vectors harboring miR-377 was constructed, and the HSCs and LX-2 were infected. Briefly, HSCs and LX-2 were cultured in McCoy's 5α medium containing 10% FBS and when they reached the exponential growth phase, 1.0×10^5 cells per well were plated in 96 plates. Next, 300 μl complete culture medium, containing recombinant lentiviruses, control lentiviruses or McCoy's 5α medium (all containing 6 μg/ml polybrene; Sigma) was added into the plates when the cells reached 50-60% confluence. Two days later, the virus-containing medium was replaced with fresh complete medium.

Histomorphology

Formalin-fixed and paraffin-embedded liver tissues were cut into about 4 μm-thick section, which were stained with hematoxylin&eosin (H&E) staining, and visualized under a microscope (Leica DM 2500).

Table 1. Physiological and biochemical properties in rat

Group	ALT (IU/L)	AST (IU/L)	PVP (mm Hg)	MAP (cm H_2O)
Sham	51.37±7.13	155.27±11.74	8.33±0.93	117.06±9.26
PH	82.36±9.59[*]	214.60±27.54[*]	15.49±2.74[*]	55.42±11.48[*]
ZP	70.44±8.06[#]	179.74±20.08[#]	12.79±2.03[*]	72.37±6.61[*]
CP	98.37±9.72[#]	290.82±33.06[#]	18.31±2.71[*]	51.06±6.69[*]

ALT, glutamic-pyruvic transaminase; AST, glutamic oxalacetic transaminase; PVP, portal venous pressure; MAP, mean arterial pressure, PH, portal hypertension; ZP, ZnPP; CP, CoPP. Values are expressed as mean ± SD, n=10 in each group. [*]$P < 0.05$, versus sham group, [#]$P < 0.05$, versus PH group.

Real time-polymerase chain reaction

RNA extraction was performed according to the TRIzol manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Synthesis of cDNAs was performed by reverse transcription reactions with 2 μg of total RNA using moloney murine leukemia virus reverse transcriptase (Invitrogen) with oligo dT (15) primers (Fermentas) as described by the manufacturer. MiR-377 level was quantified by the mirVana qRT-PCR miRNA detection kit (Ambion, Austin, USA) in conjunction with real-time PCR with SYBR Green. After circle reaction, the threshold cycle (Ct) was determined and relative miR-377 level was calculated based on the Ct values and normalized to U6 level in each sample. PCR with the following primers: α-SMA, Forward 5'-CGATTAGGCCCGTAAGGACTG-3' and Reverse 5'-GCGGCTAAGCCTGACGDACC-3'; Col1a1, Forward 5'-CGGCAGTAGGCGAAGTCCG-3' and Reverse 5'-CGGAGAGCCGTGATGGTCGAGC-3', HO-1, Forward 5'-CGAAGGAGTGCGCGAGGTG-3' and Reverse 5'-CGAAGCGGTGAAGCTGGAGA-3'; TGF-β, Forward 5'-CGAAGTTGAGGTCGATGCTG-3' and Reverse 5'-CGAAGTGGCGCTGAGTCG-ATG-3', VEGF, Forward 5'-AAGTCGGGGAAT-GAGTGGTG-3' and Reverse 5'-CGTAGAGTCG-TAGTCGAAACG-3'; CTGF, Forward 5'-CGAATG-ATCGTGAGCTAGTAAC-3' and Reverse 5'-CGA-GCGCATCAGCTGCTAGCTGA-3', MCP-1, Forward 5'-CGAGAATCCGCACGTAGCTAGTC-3' and Reverse 5'-CGAATGATCAGCTCGCTAGCTCG-3'; GA-PDH, Forward 5'-GGATTTGGTCGTATTGGG-3' and Reverse 5'-GGAAGATGGTGATGGGATT-3'.

Western blotting

Liver tissues and cells were homogenized and extracted in NP-40 buffer, followed by 5-10 min boiling and centrifugation to obtain the supernatant. Samples containing 50 μg of protein were separated on 10% SDS-PAGE gel, transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). After saturation with 5% (w/v) non-fat dry milk in TBS and 0.1% (w/v) Tween 20 (TBST), the membranes were incubated with primary antibodies (Santa Cruz Biotechnoogy, CA, USA). After three washes with TBST, The membranes were next incubated with the appropriate HRP (horseradish peroxidase)-conjugated antibody visualized with chemiluminescence (Thermo, USA).

Statistical analysis

The data from these experiments were reported as mean ± standard deviation (SD) for each group. All statistical analyses were performed by using PRISM version 5.0 (GraphPad). Intergroup differences were analyzed by one-way ANOVA. Differences with P value of < 0.05 were considered statistically significant.

Results

Serum biochemical markers

The serum level of ALT and AST in PH group was 82.36±9.59 IU/L and 214.60±27.54 IU/L, which was significantly higher than that (51.37±7.13 IU/L and 155.27±11.74 IU/L) in sham group respectively ($P < 0.05$). The serum level of ALT and AST was decreased in ZnPP treatment group and was increased in CoPP treatment group compared with PH group ($P < 0.05$). Moreover, the PVP was significantly higher and the MAP was significantly lower in the cirrhotic portal hyperpiesia rats than in the sham group ($P < 0.05$), however, the PVP and MAP had no obvious difference in the ZnPP- or CoPP-treated cirrhotic portal hyperpiesia rats (**Table 1**).

MiRNAs expression in patients with liver cirrhosis

To explore the miRNA expression profiles in liver from cirrhotic patients, we compared miRNA expression between cirrhotic patients and healthy control using miRNA microarray. The results showed that fold change greater than 2 between patients and healthy control
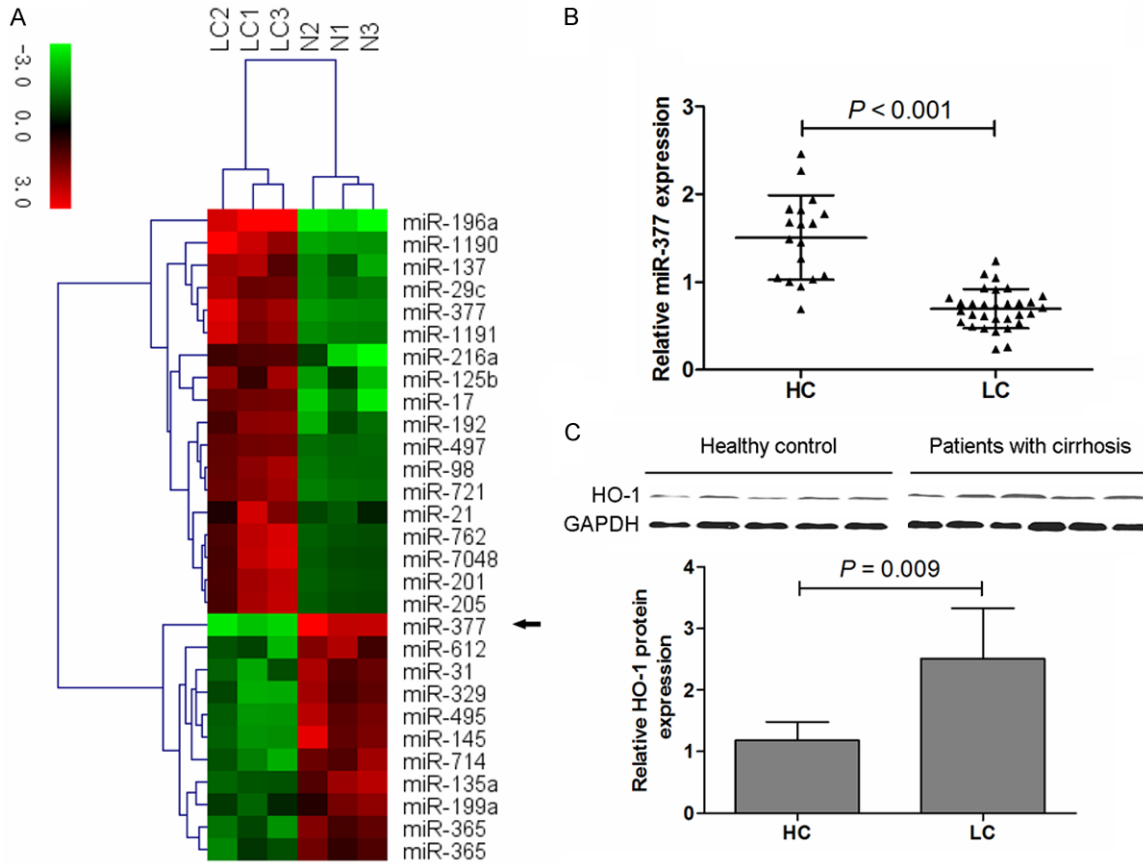
Figure 1. Microarray and hierarchical cluster analysis of the significantly regulated miRNAs in patients with liver cirrhosis and healthy control. The figure is drawn by MeV software (version 4.2.6). Correlation similarity matrix and average linkage algorithms are used in the cluster analysis. Each row represents an individual miRNA, and each column represents a sample. The dendrogram at the left side and the top displays similarity of expression among miRNAs and samples individually. The color legend at the left represents the level of miRNA expression, with red indicating high expression levels and blue indicating low expression levels (A). MiR-377 expression was examined by real-time PCR and normalized to U6 expression in Thirty-one human cirrhotic tissues and eighteen healthy control subjects (B). HO-1 protein expression was examined by western blotting and normalized to GAPDH expression in five human cirrhotic tissues and five healthy control subjects (C).

was set as the criteria to further filter the top 33 significantly dysregulated miRNAs. Among these miRNAs, the expression of 18 miRNAs was up-regulated whereas 11 miRNAs were down-regulated in patients with liver cirrhosis (**Figure 1A**). We further analyzed the expression of the top 11 down-regulated miRNAs in patients with liver cirrhosis by qRT-PCR and finally focused on miRNA-377 in our study. Subsequently, qRT-PCR analysis was performed to determine the expression level of miRNA-377 in 31 patients and 18 healthy control liver specimens. The results indicated that the expression of miRNA-377 in patients with liver cirrhosis was significantly lower than that of the healthy control group (**Figure 1B**). There is mounting evidence that HO-1 is associated

with cirrhotic portal hypertension [5, 14]. Interestingly, miR-377 regulates HO-1 protein expression during intravascular hemolysis [12]. Therefore, western blotting assay was performed to detect the expression of HO-1 in liver tissues from cirrhotic patients. As shown in **Figure 1C**, the levels of HO-1 were increased in liver tissues from cirrhotic patients as compared to those of healthy control group.

Histological analyses

Liver tissue samples from cirrhotic and sham rats were stained with H&E to examine the histopathological changes. Four weeks after the BDL operation, the bridging necrosis of hepatic cells was observed in livers of PH rats, particu-
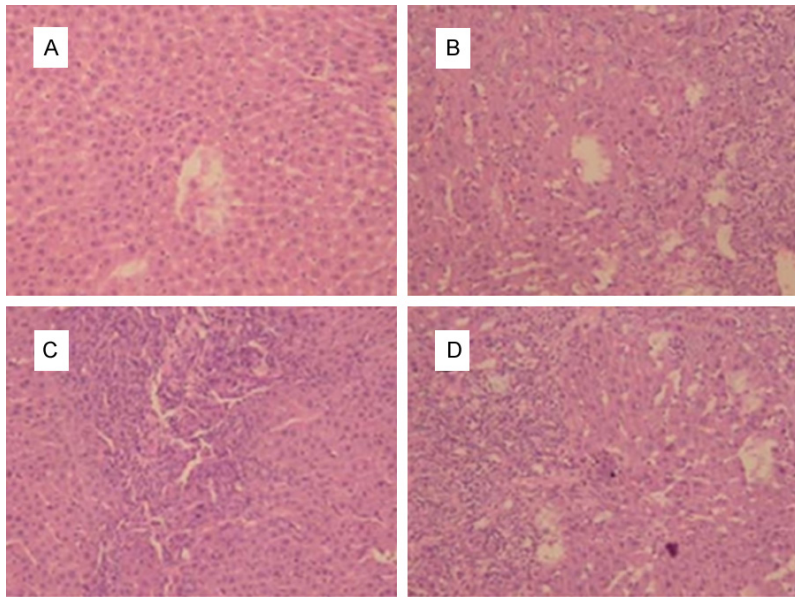
Figure 2. The sections showed typical liver tissues structures by hematoxylin and eosin (H&E) staining (200 ×). Normal liver structure (A), Liver cirrhosis (B), Liver cirrhotic rat treatment with ZnPP (C), Liver cirrhotic rat treatment with CoPP (D).

larly in portal areas, nodular regeneration of hepatocytes, collapse, and disorganization of the hepatic lobular structure, numerous lymphocytes infiltrating the portal area and around the central vein, and the formation of pseudolobules surrounded by fibrous septa. Not surprisingly, inflammatory cells and fibrous tissue were increased in CoPP treatment group as compared to that of rats in PH group. In contrast, ZnPP-treated could significantly reverse PH-induced liver tissue injury in cirrhotic portal hyperpietic rats (**Figure 2**).

MiRNA-377 via regulating HO-1 in primary HSCs and human derived LX-2 cells

In order to determine miRNAs that might regulate HO-1, the Microcosm, MiRanda, and TargetScan prediction algorithms were used in this study, we found that miRNA-377 regulated HO-1 through the predicted binding sites in its 3'-UTR. The 3'-UTR elements of HO-1 and miRNA-377 are conserved among various species, as shown by their very similar or identical sequences in rat and human (**Figure 3A**). Next, we performed luciferase assays to determine if OH-1 was directly regulated by miRNA-377. The results showed that cells were transfected with miR-377 mimic for 24 h showed approximately 70% reduced luciferase activity compared to

siRNA control group. However, mutant HO-1 3'-UTR did not show much difference as compared to control group. These results demonstrate that miRNA-377 is a regulator of HO-1 (**Figure 3B**). In addition, cell migration was measured by scratch assay. We found that microRNA-377 via targeting HO-1 regulated the cell migration in HSCs and LX-2 cell lines. MiRNA-377 gain-of function inhibited cells migration, whereas cells migration was significantly rescued by CoPP incubation in HSCs and LX-2 cell lines (**Figure 3C**). Furthermore, mRNA levels of the profibrotic markers α-SMA and collagen-1 were significantly down-regulated after transfected with miR-377 mimic, in contrast, mRNA levels of α-SMA and collagen-1 were significantly up-regulated in the present of CoPP in HSCs and LX-2 cell lines (**Figure 3D**). Similar results were found when the same experiment was repeated on HO-1 protein expression, which was significantly decreased after transfected with miR-377 mimic, and in contrast, was significantly increased in the present of CoPP in HSCs and LX-2 cell lines (**Figure 3E**).

CoPP and ZnPP regulate miRNA-377 and HO-1 in cirrhotic portal hyperpietic rats

We then tested the expression of miR-377 by qRT-PCR in cirrhotic portal hyperpiesia rats and sham rats. The expression of miR-377 was significantly blunted in cirrhotic portal hyperpiesia rats; however, miR-377 was strongly expressed in ZnPP-treated cirrhotic portal hyperpiesia rats as compared to that of rat in PH group (**Figure 4A**). In contrast to that mRNA and protein expression of HO-1 were strongly expressed in the liver tissues from HP group compared with sham group. Furthermore, mRNA and protein expression of HO-1 were significantly decreased in ZnPP treatment group and significantly higher in CoPP treatment group than in cirrhotic portal hyperpiesia group (**Figure 4B** and **4C**). To further investigate induction of fibrogenic and
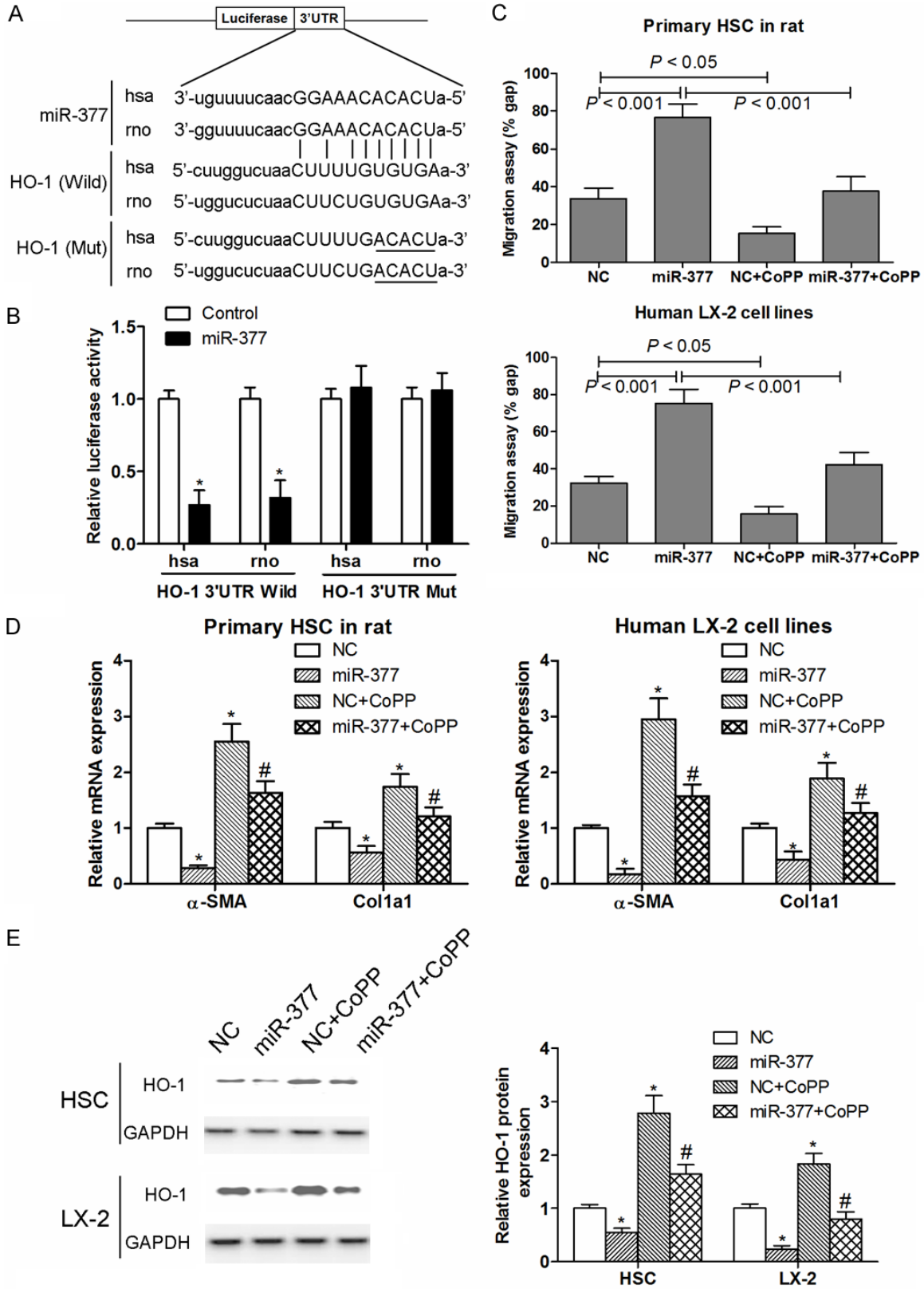
Figure 3. Schematic representation of the putative miR-377 binding site in the HO-1 3'UTR as in Targetscan (A) and luciferase activity assay (B), migration is assessed using a migration assay in HSCs and LX-2 cells (C), α-SMA and Col1a1 mRNA levels are measured by real-time PCR in HSCs and LX-2 cells (D), α-SMA and Col1a1 protein levels

are measured by western blotting in HSCs and LX-2 cells (E). *$P < 0.05$ versus control group; #$P < 0.05$ versus miR-377-transfected group. Data represent the mean ± SD of three independent experiments.
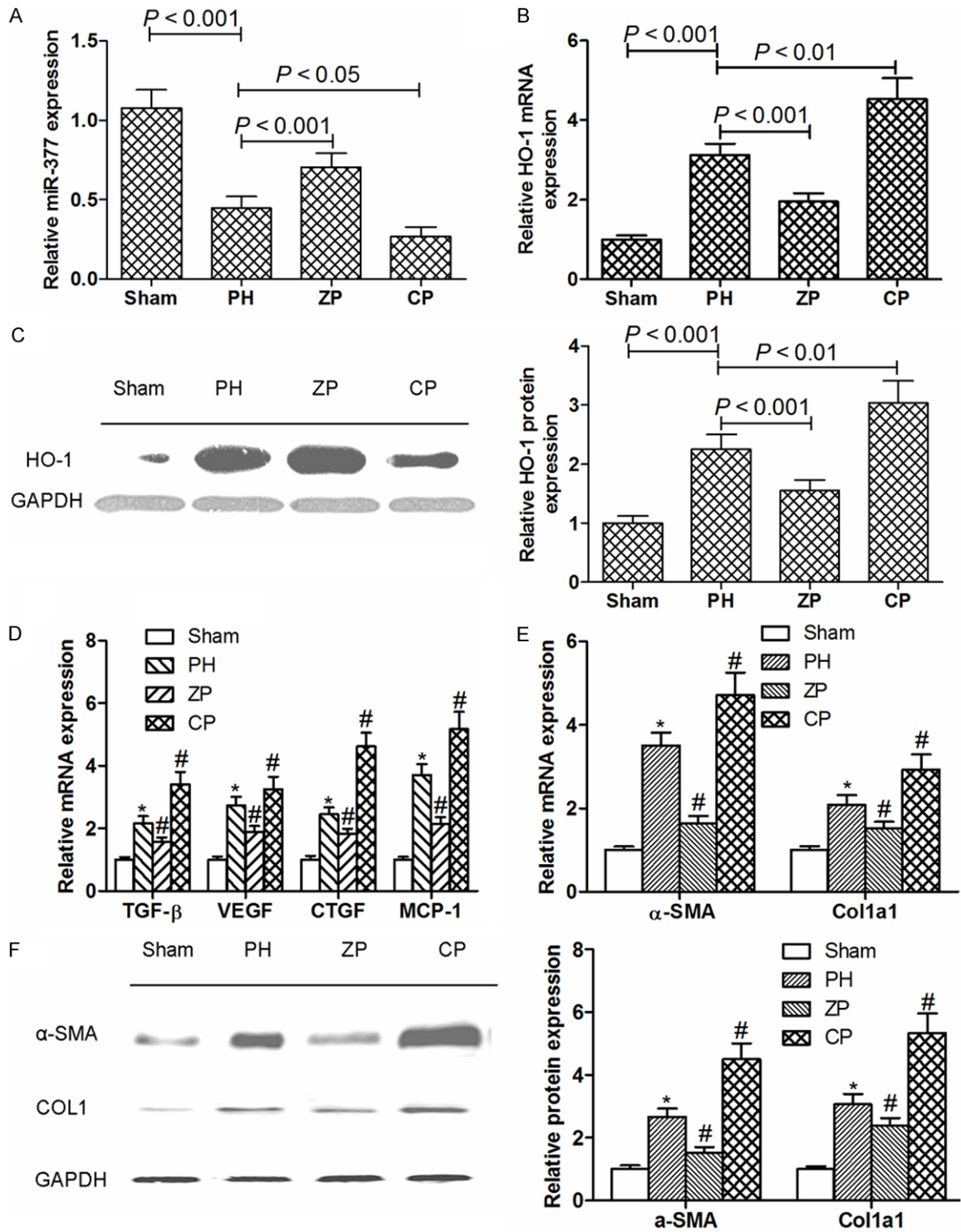

Figure 4. MiR-377 expression in liver tissues is measured by real-time PCR in rats (A). The mRNA (B) and protein (C) expression of HO-1 in liver tissues are measured by real-time PCR and western blotting respectively. TGF-β, VEGF, CTGF and MCP-1 mRNA levels are measured by real-time PCR in liver tissues (D). α-SMA and Col1a1 mRNA levels are measured by real-time PCR in liver tissues (E). α-SMA and Col1 protein levels are measured by western blotting in liver tissues (F). *$P < 0.05$ versus sham group; #$P < 0.05$ versus PH group. Data represent the mean ± SD of eight independent experiments.

inflammatory factors by BDL, the mRNA expression of TGF-β, VEGF and CTGF was measured by RT-PCR. The results showed that TGF-β, VEGF and CTGF were induced in the cirrhotic portal hyperpiesia rats and CoPP treatment cirrhotic rats. The expression of monocyte chemoattractant protein 1 (MCP-1), a chemokine promoting macrophage infiltration, was also highly induced in the cirrhotic portal hyperpiesia rats and CoPP treatment cirrhotic rats; however, ZnPP treatment could inhibited TGF-β, VEGF, MCP-1 and CTGF expression in cirrhotic portal hyperpiesia rats (**Figure 4D**). Intriguingly, mRNA and protein levels of the profibrotic markers α-SMA and collagen-1 were significantly up-regulated in the cirrhotic portal hyperpiesia rats and CoPP treatment cirrhotic rats; in contrast, ZnPP treatment could inhibit α-SMA and collagen-1 expression in cirrhotic portal hyperpiesia rats as compared to that of the PH group (**Figure 4E** and **4F**).

Discussion

Alcohol is a major cause of liver cirrhosis, and there is mounting evidence that increased hepatic vascular tone and architectural abnormalities of the liver parenchyma are the main causes in the development of portal hypertension [2]. Although the mechanisms of portal hypertension are not fully understood, carbon monoxide, a product of the heme oxygenase (HO) reaction, is thought to be one of the endogenous vasodilators in the liver [5]. Previous studies indicate that HO-1 mRNA, protein expression and its enzyme activity are up-regulated in the liver of patients with PH caused by hepatic cirrhosis [5, 14]. Our earlier studies have shown that low HO-1 expression level in kidney is an important factor for experimental hepatorenal syndrome, and Increased pulmonary HO-1 expression is an important contributor to the development of hepatopulmonary syndrome [15, 16].

In the present study, we found that the protein levels of HO-1 were increased in liver tissues from cirrhotic patients as compared to those of healthy control group. We also found that mRNA and protein expression of HO-1 were strongly expressed in the liver tissues from HP group compared with sham group. Furthermore, mRNA and protein expression of HO-1 were significantly decreased in ZnPP treatment group and significantly higher in CoPP treatment group than in cirrhotic portal hyperpiesia group. Studies have shown that the HO-1/CO system plays an important role in the control of vascular tone, and inhibition of HO-1 blocks vasodilation induced by heme [16, 17]. In addition, HO-1 is also involved in the prevention of renal failure after renal ischemia or glycerol-induced acute renal injury in rats [17, 18]. In prehepasia portal hypertensive rats, HO-1 overexpression may be a beneficial effect on liver [19, 20]. Besides, we had been demonstrated that administration of ZnPP, a strong inhibitor of heme oxygenase, elicits a marked increase in the expression of HO-1. In contrast, mRNA and protein expression of HO-1 were significantly increased in CoPP, a strong inducer of heme oxygenase, treatment group compared with cirrhotic portal hyperpiesia group. Accordingly, the serum level of ALT and AST was decreased in ZnPP treatment group and was increased in CoPP treatment group compared with PH group. These results suggested that the up-regulation of HO-1 may be involved in development and progression of portal hypertension liver cirrhosis.

The precise mechanisms via regulating HO-1 expression is induced in PH rats are still unknown, but the results here obtained lead us to believe that this induction was provoked as a consequence of miRNAs dysfunction. In our study, we examined miRNA-377 and HO-1 expressions in the liver tissues from cirrhotic patients. These results indicated that a decrease in miRNA-377 and a increase in HO-1 were observed in the liver tissues from cirrhotic patients compared with healthy control. Next, we attempted to confirm whether there was a regulatory relationship between miRNA-377 and HO-1. We provided the evidence that miRNA-377 could directly regulate HO-1 expression by binding with its 3'UTR in HSC and LX-2 cell lines, which was supported by the following reasons: miRNA-377 gain-of function significantly decreased the luciferase activity in HEK293 cells. Moreover, the overexpression of miRNA-377 decreased endogenous HO-1 expression in HSC and LX-2 cell lines. Previous studies have shown that miRNA-377 plays a critical role in the pathogenesis of diabetic nephropathy in both human cell lines and mouse models, leading to increased fibronectin production [21, 22]. Interestingly, miR-377 is involved in the regulation of HO-1 expression

during hemolysis [12]. In our work, the levels of the profibrotic markers α-SMA and collagen-1 were significantly down-regulated after transfected with miR-377 mimic, in contrast, α-SMA and collagen-1 were significantly up-regulated in the present of CoPP in HSCs and LX-2 cell lines. In vivo, α-SMA and collagen-1 were significantly up-regulated in the cirrhotic portal hyperpiesia rats and CoPP treatment cirrhotic rats, in contrast, ZnPP treatment could inhibited α-SMA and collagen-1 expression in cirrhotic portal hyperpiesia rats as compared to that of the PH group. Moreover, induction of fibrogenic and inflammatory factors by BDL were inhibited in ZnPP-treated cirrhotic portal hyperpiesia rats. Collectively, these results suggest that the down-regulation of miRNA-377 may be involved in development and progression of portal hypertension liver cirrhosis.

In conclusion, our study indicated that miRNA-377 was associated with portal hypertension in rat liver cirrhosis, and the underlying mechanism was mediated, at least partially, through the regulation of HO-1. But above all, miRNA-377 might play a potential therapeutic avenue for the treatment of portal hypertension liver cirrhosis.

Acknowledgements

Disclosure of conflict of interest

None.

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