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Influence of miR-26b on hepatic cirrhosis and portal pressure in rats with cirrhotic portal hypertension by targeting hENT1 depending on RhoA/ROCK-1 pathway

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Abstract. – OBJECTIVE: To explore the regulatory effect of micro ribonucleic acid-26b (miR-26b) on rat models of cirrhotic portal hypertension and the underlying mechanism of action.

MATERIALS AND METHODS: Common bile duct ligation (BDL) was applied to establish rat models. A total of 30 male Wistar rats were randomly divided into sham operation group (Sham group), operation group (BDL group) and miR-26b intervention group (miR-26b mimic group). Hematoxylin-eosin (HE) staining assay was performed to detect pathological characteristics of rat liver tissues in each group. The portal venous pressure in each group was then determined. The levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in rat serum were measured *via* serological test. Kits were used to detect serum levels of hyaluronic acid (HA), procollagen III peptide (PCIII) and laminin (LN) in rats. Western blotting was utilized to detect the protein levels of human equilibrative nucleoside transporter 1 (hENT1), ras homolog gene family, member A (RhoA) and Rho-associated coiled-coil-containing kinase protein-1 (Rock-1).

RESULTS: In comparison with Sham group, BDL group had significantly increased portal venous pressure and protein levels of hENT1, RhoA and ROCK-1, and elevated levels of AST, ALT, HA, PCIII and LN in rats. Compared with those in BDL group, the portal venous pressure and protein levels of hENT1, RhoA and ROCK-1 were overtly reduced, while the levels of AST, ALT, HA, PCIII and LN declined in miR-26b mimic group.

CONCLUSIONS: MiR-26b mimics played a role in the treatment of rats with cirrhotic portal hypertension by targeting hENT1 to inhibit the RhoA/ROCK-1 signaling pathway.

Key Words:
miR-26b, RhoA/ROCK-1, hENT1, Hepatic cirrhosis, Portal hypertension.

Introduction

Hepatic cirrhosis is a chronic liver disease characterized by diffuse fibrosis of liver tissues and formation of pseudolobules and regenerative nodules. The main pathological manifestations are portal hypertension and liver function damage[1-3]. Portal hypertension, the most common complication of decompensated hepatic cirrhosis, is able to cause upper gastrointestinal hemorrhage and even cirrhosis death. As early as the 1980s, the main theory of pathogenesis of hepatic cirrhosis was "reverse blood flow", that is, remodeling of pseudolobules and blood vessels. With in-depth research, researchers have found that the pathogenesis of hepatic cirrhosis also includes the occurrence of portal hypertension, vascular resistance increase and activation of hepatic stellate cells (HSCs)[4-6]. Considering the pathogenesis of cirrhotic portal hypertension, drugs constricting splanchnic vessels and lowering angiotasis are mainly used in clinical practice, greatly reducing the mortality rate of patients with hepatic cirrhosis. However, the selectivity of existing drugs is poor and drug resistance occurs easily. Therefore, it is urgent to find drugs that can constantly and stably reduce portal venous pressure.

For portal hypertension occurring in early hepatic cirrhosis, abnormal activation of HSCs is an important factor. HSCs secrete large amounts of collagen, which accumulates in the live and causes liver fibrosis[7]. Currently, nucleoside analogs inhibiting the activation of HSCs are therefore considered as the primary method for the treatment of cirrhotic portal hypertension. Human equilibrative nucleoside transporter 1 (hENT1) with gene name as SLC29A1 contains approximately 465 residues, which bind to the low affinity nucleosides and transfer to the lower concentration side of the membrane[8]. Ras homolog

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gene family member A (RhoA), as an important member of the small G-protein Rho family, constitutes the RhoA/Rho-associated coiled-coil-containing kinase protein-1 (Rock-1) signaling pathway together with the downstream effector Rho-associated kinase (ROCK), playing an important role in the fibrosis of liver tissues[9,10].

Micro ribonucleic acids (miRNAs), a class of endogenous highly-conserved single-stranded non-coding RNAs with about 20-24 nucleotides in length, are hotspots in the field of medicine in recent years. MiRNAs are reported to be involved in the growth, proliferation, differentiation and apoptosis of cells[11]. Gao et al[12] demonstrated that miR-26b targetedly regulates hENT1 through luciferase reporter gene assay. However, there is no report on the regulatory role of miR-26b in rat models of cirrhotic portal hypertension by targeting hENT1 depending on the RhoA/ROCK-1 signaling pathway. Therefore, we aim to investigate the regulatory effect of miR-26b on rats with cirrhotic portal hypertension and its mechanism of action.

Materials and Methods

Reagents

The reagents used in this study were as follows: 4% paraformaldehyde (purchased from Beijing Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China), bicinchoninic acid (BCA) protein concentration assay kits (Beyotime, Shanghai, China), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) kits (Sigma-Aldrich, St. Louis, MO, USA), hENT1, RhoA and ROCK-1 primary antibodies (Abcam, Cambridge, MA, USA) and horseradish peroxidase (HRP)-labeled secondary antibodies (CST, Danvers, MA, USA).

Instruments

The reagents used in this study were as follows: A thermostatic water bath (bought from Fuhua Instrument Co., Ltd., Shenzhen, China), a micro-vortex instrument (Shanghai Huxi Analysis Instrument Factory Co., Ltd., Shanghai, China), a -80°C ultra-low-temperature refrigerator (Haier, Qingdao, China), a high-temperature sterilizer (Yamato Scientific, Shanghai, China) and a biochemical analyzer (Roche, Basel, Switzerland).

Animals

Healthy male Wistar rats weighing (200±10) g were randomly divided into three groups: sham operation group (Sham group, n=10), operation group (BDL group, n=10) and miR-26b intervention group (miR-26b mimic group, n=10). This study was approved by the Animal Ethics Committee of Fudan University Animal Center (Fudan, China).

Methods

Establishment of Rat Models of Hepatic Fibrosis Portal Hypertension

All rats fasted for 12 h underwent surgery by surgical instruments sterilized by high-temperature. The chest was scissored to expose the liver, and a short duct with a certain length of the common bile duct near the portal vein was then gently ligated. After that, the skin was sutured, followed by disinfection with iodine. The modeling method was simple, safe and non-toxic, with a short experimental period. The rats showed consistent pathological features with those of human micronodular cirrhosis, and were considered as ideal models for studying portal hypertension.

Detection of Pathological Changes in Rat Liver via Hematoxylin-Eosin (HE) Assay

Liver tissues collected from each group were immersed in paraformaldehyde solution, dehydrated, embedded with paraffin and sectioned before HE staining (Solarbio, Beijing, China). Hematoxylin was used to stain cell nucleus, and eosin was utilized to stain cytoplasm. Next, the stained cells were mounted, photographed and analyzed using a microscope.

Measurement of Serum ALT and AST Levels in Rats via Serological Test

Blood samples were taken from each group of rats and then centrifuged. The serum was collected and kept at room temperature for 2 h before centrifugation. The supernatant was taken and the content of ALT and AST was determined using the automatic biochemical analyzer (Roche Modular DPP, Basel, Switzerland).

Determination of Hyaluronic Acid (HA), Procollagen III Peptide (PCIII) and Laminin (LN) Levels in Rat Serum Through Kits

The levels of HA, PCIII and LN in the serum of each group of rats were measured according to the instructions of kits (Promega, Madison, WI, USA).

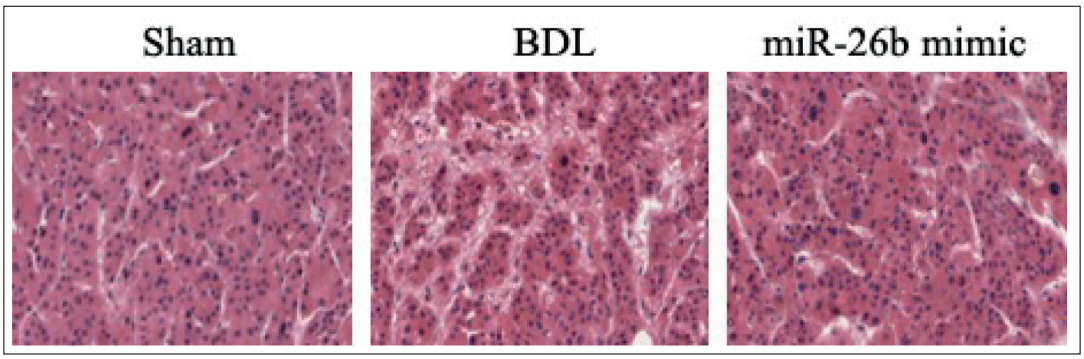

Figure 1. Pathological samples of rat liver tissues (×40).

Detection of Protein Levels of hENT1, RhoA and ROCK-1 in Rat Liver Tissues by Western Blotting Assay

Liver tissues of rats in each group were extracted by protein lysis solution. After centrifugation, the supernatant was retained. The BCA protein concentration assay kit was employed for the determination of protein concentration. After denaturation in water bath at 100°C, the protein samples were loaded and subjected to electrophoresis followed by transferring to the membranes. Next, it was incubated with hENT1, RhoA and ROCK-1 primary antibodies and secondary antibodies, respectively. Finally, the membranes were subjected to color development via diaminobenzidine (DAB) color-developing solution (Sigma-Aldrich, St. Louis, MO, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 analysis software (SPSS Inc., Chicago, IL, USA) was used for analysis. The results were expressed as mean ± standard deviation. Analysis of variance (ANOVA) or independent-samples *t*-test was applied for each group. One-way ANOVA test was followed by LSD (Least Significant Difference) post-hoc test. $p<0.05$ suggested that the difference was statistically significant.

Results

Staining Results of Pathological Sections

In the Sham group, the rat liver showed ruddy color, and smooth surface without adhesion. In the BDL group, the surface of rat liver was greasy and adhesive, indicating that rat models of hepatic cirrhosis were successfully prepared. HE staining was conducted to detect the pathological changes of the rat liver in each group. The results demonstrated that the hepatocytes in Sham group were orderly arranged with tight intercellular space. In the BDL group, hepatocytes' arrangement was disordered, cells were fused, and interstitial cells were infiltrated by inflammatory cytokines. Compared with that in BDL group, the pathologic condition of hepatic cirrhosis of rats in miR-26b mimic group showed a significant improvement (Figure 1).

Portal Venous Pressure Value Measured

Portal venous pressure is mostly caused by hepatic cirrhosis. Once blocked, the blood flow in veins will flow back to the inferior vena cava through the liver, causing excessive venous pressure or even acute upper gastrointestinal hemorrhage and death in severe cases. The values of portal venous pressure of three groups of rats were measured here. We found that the portal venous pressure was significantly higher in BDL group than in Sham group ($^{**}p<0.01$), while it was overtly decreased in miR-26b mimic group compared with that in BDL group ($^{\#}p<0.05$) (Figure 2).

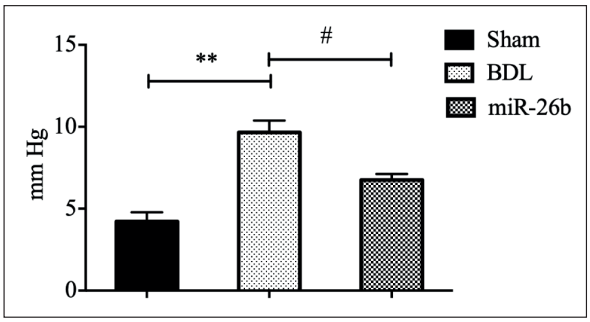

Figure 2. Portal venous pressure of rats ($^{**}p<0.01$, $^{\#}p<0.05$).

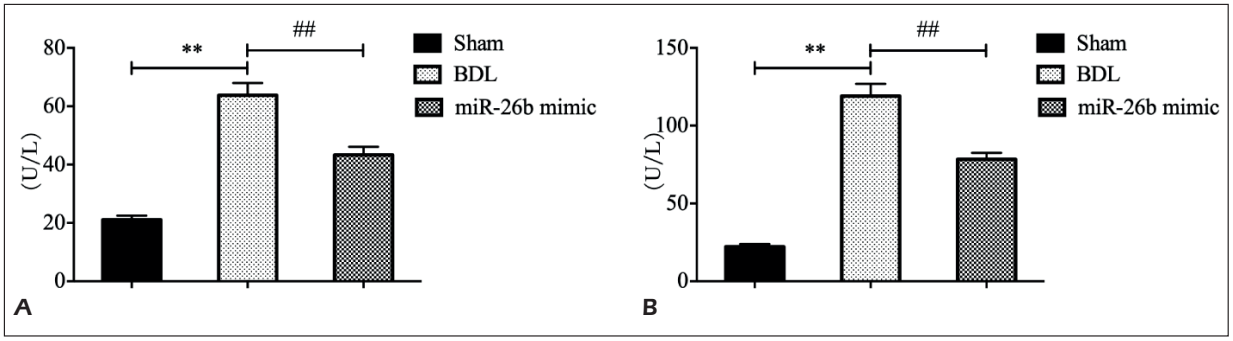

Figure 3. AST and ALT levels in rat serum. **A**, AST level (**p<0.01, ##p<0.01), **B**, ALT level (**p<0.01, ##p<0.01).

MiR-26b Mimics Reduced AST and ALT Levels in Rat Serum

The continuous increase of AST and ALT indicates a progressive liver disease. According to Figure 3, the levels of AST (Figure 3A) and ALT (Figure 3B) in rat serum were clearly elevated in BDL group compared with that in Sham group, while miR-26b mimic group had evidently lowered levels of AST and ALT in comparison with BDL group (**p<0.01, ##p<0.01).

MiR-26b Mimics Lowered HA, PCIII and LN Levels

HA reflects the amount of fiber and the damage degree of the liver. PCIII is closely related to the activity degree of liver fibrosis formation. LN is positively associated with the degree of liver fibrosis and portal venous pressure. These three markers of liver fibrosis were detected in this study. The results revealed that the levels of HA, PCIII and LN in rat serum in BDL group were evidently higher than those in Sham group (*$p < 0.05$, **$p < 0.01$, *$p < 0.05$), while they were significantly decreased after treatment with miR-26b mimics (##$p < 0.01$, ##$p < 0.01$, #$p < 0.05$) (Table I). Our data suggested that miR-26b mimics could inhibit liver fibrosis.

MiR-26b Mimics Decreased the Protein Levels of hENT1, RhoA and ROCK-1

To further investigate the regulatory mechanism of miR-26b in hepatic cirrhosis and portal pressure in rats with cirrhotic portal hypertension, Western blotting assay was carried out to detect the expression levels of hENT1, RhoA and ROCK-1. The results (Figure 4A) showed that compared with those in Sham group, the protein levels of hENT1, RhoA and ROCK-1 were clearly increased in BDL group compared to the Sham group (*p<0.05, **p<0.01, *p<0.05). However, a significant decline of these proteins was observed in miR-26b mimic group compared with those in BDL group (#p<0.05, #p<0.05, ##p<0.01) (Figure 4B).

Discussion

Hepatic cirrhosis results from repeated interactions of chronic liver diseases caused by multiple factors. The inducing factors of the disease include alcohol, virus, fat accumulation, autoimmunity, etc. Although no significant abnormal symptoms were detected in the compensated stage during the progression of hepatic cirrhosis, hepatic injury and portal hypertension easily occurred in the decompensated stage and were mainly represented as ascites, gastrointestinal hemorrhage, collateral circulation or hepatic encephalopathy[13-15]. Portal hypertension is the most common complication of hepatic cirrhosis. With the development of biological cell experiments, the researches on hepatic cirrhosis have entered a new dimension. It is reported that the activation of HSCs and the secretion of collagen

Table I. Levels of HA, PCIII and LN in rat serum.

Group	HA	PCIII	LN
Sham group	130.56±3.12	80.34±7.51	30.68±4.86
BDL group	267.34±5.71*	230.84±7.92**	67.69±5.72*
MiR-26b mimic group	198.33±6.34##	189.36±8.13##	±4.75#

Notes: * and #: p<0.05,** and ##: p<0.01, * and **: comparison between BDL group and Sham group, # and ##: comparison between miR-26b mimic group and BDL group.

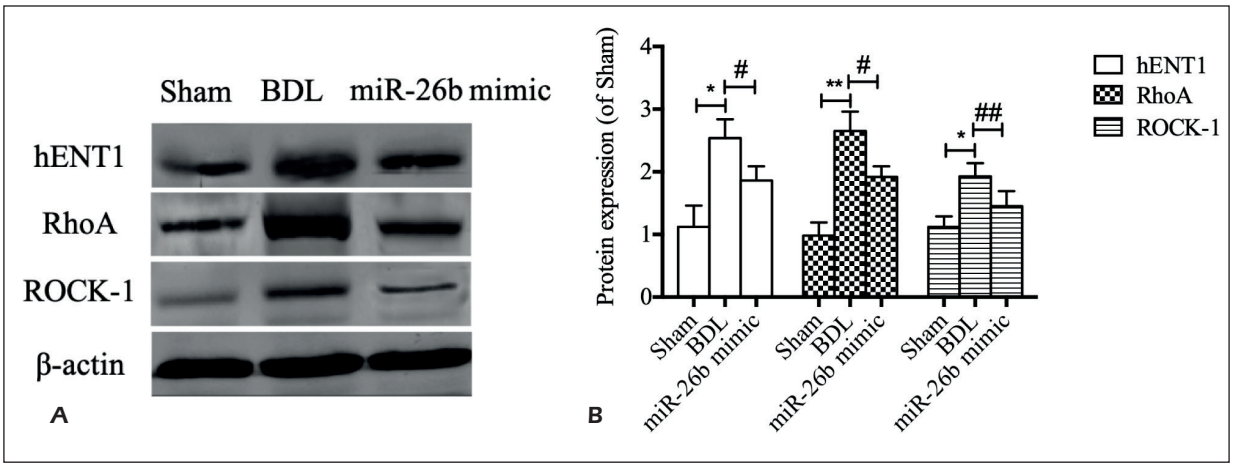

Figure 4. Protein levels of hENT1, RhoA and ROCK-1. **A**, Western blotting bands, **B**, Western blotting band cartogram (*p<0.05, **p<0.01, $^{\#}p$<0.05 and $^{\#\#}p$<0.01).

matrix are crucial for the occurrence and development of portal hypertension. HSCs are thought to be the main source of extracellular matrix and usually stay quiescent under normal conditions. Activated HCSs could differentiate into fibroblasts with the ability of proliferation and secretiation of collagen matrix, thus resulting in liver injury and aggravating liver fibrosis[16]. Currently, nucleoside analogs are clinically used to inhibit the proliferation of HSCs, thereby suppressing the secretion of collagen matrix in HSCs. Nucleoside is the basic unit of the body and involved in every physiological and biochemical process in the body. HENT1 is the regulatory target of nucleoside analogs. Borbath et al[17] investigated 43 cases of cholangiocarcinoma and found that the expression level of hENT1 directly affected the survival rate of cholangiocarcinoma patients received gemcitabine treatment. In view of the regulatory effects of nucleosides, miRNAs are expected to become novel molecular-targeted drugs with non-toxic or side effects against cirrhotic portal hypertension. The main mechanism of miRNAs is to repress the translation of target genes into proteins or promote the degradation of target genes. He et al[18] discovered that miR-351 was lowly expressed in normal HSCs and it triggered and aggravated liver fibrosis through the target receptor vitamin D receptor after infection with virus. However, the occurrence and development of liver fibrosis were inhibited and alleviated to some extent if the expression of miR-351 was suppressed. By using the miRNA microarray technology, Hyun et al[19] found that the expression levels of miR-378 family members (miR-378a-3p, miR-378b and miR-378d) were reduced in mouse models of carbon tetrachloride-induced hepatic cirrhosis. The proliferation of HSCs was inhibited after transfection with miR-378a-3p mimics, and a further study revealed that miR-378a-3p inhibited the activation and proliferation of HSCs by targeting Gli3 target, thereby repressing liver fibrosis. Increasing researches have manifested that miRNAs might influence the development of hepatic fibrosis by inhibiting the proliferation and activation of HSCss, suggesting that miRNAs might be the most promising drugs for the treatment of liver fibrosis. The RhoA/ROCK-1 signaling pathway is a ubiquitous signal transduction pathway in the body, which is involved in various biological processes such as morphogenesis, adhesion, migration and proliferation of cells. Although the RhoA/ROCK-1 signaling pathway has been reported to be related to multiple fibrotic diseases, researches on its role in liver fibrosis are rare[20]. The expression of ROCK-1 is evidently increased when fibrosis was occurred, while remarkably decreased after treatment with existing clinical drugs for liver fibrosis inhibition. Above evidence further confirmed that the RhoA/ROCK-1 signaling pathway might play an important role in liver fibrosis. In this study, rat models of cirrhotic portal hypertension were prepared using common BDL. HE staining was performed on liver tissues to observe their pathological changes, and the results showed that the pathologic condition of the liver in miR-26b mimic group was significantly improved. MiR-26b mimics reduced both the portal venous pressure and the level of collagen matrix in rats with hepatic cirrhosis, as well as decreased the occurrence of liver fibrosis. To further explore the regulatory mechanism of miR-26b, the protein levels of hENT1, RhoA, and ROCK-1 were measured in this study. Our data revealed that the protein levels of hENT1, RhoA and ROCK-1 of rat liver tis-

sues in BDL group were clearly higher than those in Sham group and miR-26b mimic group, while overtly decreased after miR-26b mimic treatment. Our results indicated that miR-26b mimics targeted hENT1 to reduce portal venous pressure and suppress the secretion of collagen matrix through the RhoA/ROCK-1 signaling pathway, thereby relieving liver fibrosis in rats.

Conclusions

We manifested that miR-26b might play a regulatory role in rats with cirrhotic portal hypertension, and its mechanism of action might be related to the inhibition of the activation of RhoA/ROCK-1 signaling pathway by targeting hENT1. Above results provided new ideas and methods for the continuous development of drugs treating hepatic fibrosis in the future.

Conflict of Interests

The authors declared no conflict of interest.

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