MicroRNA-122 mediates the protective effect of baicalin on liver fibrosis

Min Zhang, Dexin Wang, Baofeng Ma

Department of Medicine, The Sixth People’s Hospital of Qingdao, Qingdao, Shandong, China

Received May 30, 2016; Accepted August 3, 2016; Epub October 15, 2016; Published October 30, 2016

Abstract: As the most abundant miRNA in the liver, miR-122 has been suggested to be involved in multiple hepatic disorders such as hepatic carcinoma, HCV infection, and non-alcoholic fatty liver disease. However, its role in liver fibrosis is not fully understood. In this study, we tested the potential involvement of miR-122 in liver fibrosis and baicalin-induced improvement of fibrotic changes. Twenty liver fibrosis patients and twenty matched healthy volunteers were enrolled in this study. Serum ALT, AST, and miRNA levels were detected. Liver fibrosis animal model was induced by intraperitoneal injection of CCl₄. Patients with liver fibrosis had a reduced serum level of miR-122, accompanied with elevated serum ALT and AST levels. Baicalin dose-dependently modulated CCl₄-induced liver damage and collagen deposition, and increased miR-122 expression in both liver and serum. In consistency, KLF6, a direct target of miR-122 which contributes to fibrogenesis, reduced in baicalin-treated mice with hepatic fibrosis. Our study suggests the therapeutic effect of baicalin on hepatic fibrosis may involve miR-122 and its target gene KLF6.

Keywords: Liver fibrosis, baicalin, microRNA, miR-122, KLF6

Introduction

Liver fibrosis is a healing process of chronic liver injury caused by a variety of etiologies including viral hepatitis, chemical exposure, nonalcoholic steatohepatitis, and autoimmune disease [1, 2]. The mechanisms involve injury-induced hepatocyte apoptosis, cholangiocyte proliferation, inflammatory response, and activation of fibroblast, stem cell, and progenitor cell [3]. However, the exact mechanisms are not fully understood. MicroRNAs (miRNAs), as important endogenous regulators of gene expression, participate in many pathological conditions including hepatic disorders [4]. miRNAs are frequently disregulated and are considered as novel tissue-based markers for the diagnosis and monitoring of several liver diseases [5]. miR-122 is highly expressed in the liver, accounting for 70% of the total miRNAs in the liver [6]. It has been suggested that miR-122 is involved in multiple liver diseases such as hepatic carcinoma [7, 8], HCV infection [9, 10], and non-alcoholic fatty liver disease [11-13].

Baicalin (7-d-glucuronic acid,5,6-dihydroxyflavone), is a bioactive flavone found in several species in the genus Scutellaria such as Scutellaria baicalensis and Scutellaria lateriflora. It is the major component responsible for the anxiolytic effects of Scutellaria baicalensis and Scutellaria lateriflora [14]. It also has many other biological functions including hepatoprotective effects [15-19]. Baicalin has been reported to improve liver injury and fibrosis by suppressing TGFβ1, a major fibrogenic inflammatory cytokine, and activating Peroxisome proliferator-activated receptor gamma (PPARγ), a nuclear receptor with protective effect in fibrosis [15]. In the current study, we examined the role of miR-122 in mediating the protective effect of baicalin on liver fibrosis.

Material and methods

Patients

Twenty liver fibrosis patients diagnosed by ultrasonographic examination in Qingdao No. 6 People’s Hospital were enrolled in this study. Exclusion criteria were pregnancy, tumor, recent infection (in 3 months), and autoimmune diseases. Twenty age-, race-, and sex-matched healthy volunteers were recruited as controls. Blood was collected after overnight fasting and
miR-122 in liver fibrosis

serum was isolated by centrifugation at 2000× g for 15 min. The study was approved by the hospital ethics committee.

Animals and liver fibrosis induction

ICR/HaJ mice were purchased from Shanghai JieSiJie Laboratory Animal Co., Ltd. (Shanghai, China). Seventeen mice were randomly divided into 2 groups: control group and liver fibrosis group. For the induction of liver fibrosis, mice were i.p. injected with 0.1 ml/kg 2% carbon tetrachloride (CCl₄, Aladdin Industrial Corporation, Shanghai, China) in corn oil, twice weekly for 6 weeks. After 6 weeks of induction, mice in liver fibrosis group were further divided into 4 groups: CCl₄ group, low dose baicalin + CCl₄ group, medium dose baicalin + CCl₄ group, and high dose baicalin + CCl₄ group. For baicalin treatment, mice were gavaged with indicated doses of baicalin in 1% Tween-80 solution. The grouping and treatment information was listed in Table 1. All animal studies have been approved by the Institutional Animal Care and Use Committee (IACUC).

Serum ALT and AST measurement

Blood isolated from patients or animals was centrifuged at 2000× g for 15 min and serum was collected for ALT and AST detection. ALT and AST levels were detected using commercial kits from Jiancheng Institute of Biotechnology (Nanjing, China) following the instructions of the manufacturer.

Total RNA isolation

For liver RNA isolation, liver homogenate was used for total RNA isolation using TRIzol (Invitrogen, Grand Island, NY) as instructed by the manufacturer. For RNA isolation from the serum, 20 μL serum was mixed with 20 μL solution A (0.2 mol/LTris-HCl, pH 7.5, 25 mmol/L EDTA, 0.3 mol/L NaCl, 2% SDS, 0.1% DEPC) and boiled for 3 min. After centrifugation at 14000 rpm for 3 min, supernatant was collected for reverse transcription.

Real-time PCR detection of miRNA

The first strand cDNA was synthesized using miRcute miRNA First-strand cDNA kit (Tiangen, Beijing, China; Cat #KR201) and the real-time PCR was performed using miRcute miRNA qPCR Detection kit (Tiangen, Beijing, China; Cat #FP401) as instructed by the manufacturer. The program was as follows: 94°C 2 min, 40 circles of 94°C 10 sec and 66°C 20 sec.

Real-time PCR detection of liver KLF6 mRNA

Total RNA in the liver was extracted using TRIzol (Invitrogen, Grand Island, NY) and cDNA was synthesized by using Takara Reverse Transcriptase M-MLV (Takara Co., Ltd., Japan; Cat #2641A) following the manufacturers’ instructions. Real-time PCR was performed using a SuperReal PreMix Plus (SYBR Green) kit (Tiangen, Beijing, China; Cat #FP205-1) on a StepOnePlus™ Real-Time PCR System as instructed by the manufacturers. The KLF6 mRNA was amplified using the following primers (Forward: TGACAAGGTAATGGCGACGC; Reverse: CACCGGTATGCTTTCGGAAGTG) and PCR program: 95°C 15 min, 40 circles of 95°C 10 sec and 66°C 20 sec.

3.7 HE staining

HE staining of liver tissue sections were performed as previously described [20]. Briefly, Paraffin embedded sections were stained with hematoxylin for 10 min and 0.5% eosin for 3 sec. Images were captured at 200× magnifications.

Table 1. Grouping and treatment information

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>CCl₄</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>Low dose baicalin + CCl₄</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>Medium dose baicalin + CCl₄</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>High dose baicalin + CCl₄</td>
<td>3</td>
</tr>
</tbody>
</table>

Notes: CCl₄, carbon tetrachloride; i.p., intraperitoneal injection.
miR-122 in liver fibrosis

Van-Gieson staining for collagen fibers

Paraffin embedded sections were stained with hematoxylin for 20 min. After washes, sections were then stained with Van Gieson solution, followed by dehydration and clarification. Images were captured at 200× magnifications.

Statistical analysis

All data are shown as means ± standard deviation and statistically analyzed using one-way analysis of variance or Student’s t-test where appropriate. The statistical analyses were performed using GraphPad Prism 5.0 software. P<0.05 was considered as statistically significant.

Results

ALT and AST levels in patients with liver fibrosis

Growing evidence suggests miRNAs play an important role in liver pathology [4]. MicroRNA-122 (miR-122) is abundantly expressed in the liver and has been suggested to be involved in hepatic carcinoma [7, 8], HCV infection [9, 10], and non-alcoholic fatty liver disease [11-13]. However, its role in liver fibrosis is not well-defined [21]. To investigate the potential involvement of miR-122 in liver fibrosis, we recruited 20 liver fibrosis patients and 20 healthy controls. Serum was isolated from the peripheral blood and used for the detection of ALT and AST. Results showed a mild to moderate increase of both ALT and AST in the patients with liver fibrosis (Figure 1A and 1B), suggesting liver cell injury in patients with liver fibrosis.

Serum miR-122 reduced in liver fibrosis

We next examine the level of miR-122 in the serum. As depicted in Figure 2, there was a ~5-fold decrease of serum miR-122 in liver fibrosis patients (P<0.01). This result suggests a potential involvement of miR-122 in the pathogenesis of liver fibrosis.
miR-122 in liver fibrosis

As shown in Figure 3, CCl4 injected resulted in elevation of serum ALT and AST. All doses of baicalin (100 mg/kg, 200 mg/kg, and 400 mg/kg) restored serum AST level (P<0.01). However, no significant changes in serum ALT were observed in baicalin-treated mice although there was a trend to decrease ALT in baicalin-treated mice (Figure 3).

Effect of baicalin on hepatic HYP level

Liver hydroxyproline (HYP) is an important marker for liver fibrosis. We therefore measured HYP content in the liver. Compared to normal group, liver HYP content significantly increased in the CCl4-treated mice (P<0.01). Treatment with 400 mg/kg baicalin suppressed CCl4-induced elevation of liver HYP, suggesting the therapeutic effect of baicalin on CCl4-induced liver fibrosis (Figure 4).

Effect of baicalin on hepatic histopathology

HE staining of liver sections in control group revealed that the hepatic lobules had clear structure and the hepatocyte tubes arranged orderly and closely. No inflammatory cell infiltration and hepatocyte death were observed (Figure 5A). In contrast, CCl4 group showed destruction of some hepatic lobules, disordered hepatocyte tubes, inflammatory cell infiltration, and hepatocyte swelling and necrosis (Figure 5B). Baicalin dose-dependently reversed those pathological changes induced by CCl4 (Figure 5C-E). In consistency, the VG staining of liver from control group displayed a normal hepatic lobule structure (Figure 6A). Livers from CCl4 group showed disordered hepatocyte tubes and plenty collagen deposition (Figure 6B), while baicalin treatment especially the high dose baicalin reduced CCl4-induced collagen deposition (Figure 6C-E).
miR-122 in liver fibrosis

Baicalin increased serum miR-122 in CCl₄-induced liver fibrosis

In consistency with human data, mice with liver fibrosis showed a marked decrease in serum miR-122 level. Both mid dose and high dose baicalin significantly increased serum miR-122 level. Low dose baicalin treatment slightly elevated serum miR-122 although did not reach statistical significance (Figure 7).

Effect of baicalin liver miR-122 and KLF6 mRNA expression

We then detected the level of miR-122 in the liver of baicalin-treated mice. As illustrated in Figure 8A, miR-122 expression markedly reduced in the liver of CCl₄-treated mice while baicalin especially at high dose dramatically upregulated the expression of miR-122 in the liver. KLF6 is a direct target of miR122 and con-
miR-122 in liver fibrosis

Figure 7. Effect of baicalin on serum miR-122 in CCl₄-treated mice. Serum isolated from mice treated with 2 mg/kg CCl₄, or 2 mg/kg CCl₄ + indicated doses of baicalin for 6 weeks was used for the detection of miR-122 expression level. U6 was used as an endogenous control. *P<0.05, **P<0.01 vs. normal; #P<0.05, ##P<0.01, ###P<0.001 vs. CCl₄. CCl₄, carbon tetrachloride.

miR-122 is involved in many liver disorders such as hepatic carcinoma, HCV infection, and non-alcoholic fatty liver disease. In this study, we tested the potential involvement of miR-122 in liver fibrosis and baicalin-induced improvement of fibrotic changes. Serum level of miR-122 reduced in both patients with liver fibrosis and mice with CCl₄-induced hepatic fibrosis. Baicalin treatment improved liver fibrosis, restored CCl₄-induced miR-122 down-regulation, and elevated expression level of KLF6. Our study suggests the therapeutic effect of baicalin on hepatic fibrosis may involve miR-122 and its target gene KLF6.

Discussion

Despite of recent advances in hepatic disorders, current therapies for liver fibrosis are not fully understood. The miRNAs, endogenous small non-coding RNAs, play an important role in regulating hepatic functions [4]. As the most abundant miRNA in the liver, miR-122 is involved in many liver disorders such as hepatic carcinoma, HCV infection, and non-alcoholic fatty liver disease. In this study, we demonstrated the potential involvement of miR-122 in liver fibrosis and baicalin-induced improvement of fibrotic changes. Serum level of miR-122 reduced in both patients with liver fibrosis and mice with CCl₄-induced hepatic fibrosis. Baicalin treatment improved liver fibrosis, restored CCl₄-induced miR-122 down-regulation, and elevated expression level of KLF6. Our study suggests the therapeutic effect of baicalin on hepatic fibrosis may involve miR-122 and its target gene KLF6.

miRNAs are a class of evolutionarily conserved, small non-coding RNAs. They suppress the translation and enhance the degradation of target mRNAs via a partially complementary binding to the 3’ untranslated region (UTR) [22]. There are over 1000 miRNAs have been identified in humans with more than half of them have been experimentally validated [23]. miR-122 is the most abundant miRNA in the liver, accounting for 70% of the liver’s total miRNAs [6]. Mice deficient for miR-122 develop temporally controlled steatohepatitis, fibrosis, and hepatocellular carcinoma [6].

In this study, we used CCl₄-induced liver fibrosis as a model to assess the role of miR-122 in hepatic fibrosis and baicalin-induced therapeutic effect. CCl₄ is a well-known for its toxicity to the liver and has been widely used as a hepatotoxicant to establish acute [24] or chronic [25] liver injury models in rodents. CCl₄ generates trichloromethyl free radicals by cytochrome P450 2E1 (CYP2E1) in vivo [26, 27]. It also enhances the release of many inflammatory cytokines such as tumor necrosis factor α (TNFα) and transforming growth factor β1 (TGFβ1) [28, 29]. TGFβ1 may then induce the production and deposition of collagen, which finally results in severe hepatic fibrosis [30]. In CCl₄-treated mice, we found a significant reduction of miR-122, which is accompanied by hepatocyte injury, collagen depositions in the liver and the increases of hepatic HYP and serum ALT and AST. There was also an increase of the mRNA expression of KLF6, a direct target of miR-122. These CCl₄-induced changes were significantly modulated by the treatment of baicalin.

Baicalin is a bioactive flavone found in Scutellaria. It has been reported to have effects on a number of liver diseases such as hepatic fibrosis [15-19]. It improves liver injury and fibrosis by suppressing TGFβ1 and activating Peroxisome proliferator-activated receptor gamma PPARγ [15]. However, whether miRNAs are involved in the protective effect of baicalin has not been well-characterized. In this study, we demonstrated that baicalin treatment dose-dependently restored the expression of miR-122 in the serum and liver of CCl₄-treated mice. In addition, baicalin also reduced KLF6, a fibrogenic nuclear protein that is directly targeted by miR122 [6]. These results indicate miR-122 may be an important mediator for baicalin-
induced hepatoprotective effect. The limitations of this study include: the cause-and-effect relationships between liver fibrosis and miR-122 was not further investigated; we did not test whether baicalin-mediated hepatoprotective effect is dependent on KLF6. It requires further investigations to address these questions.

In summary, our study indicates miR-122 may serve as a marker for hepatic fibrosis and it may mediate the hepatoprotective effects of baicalin on liver fibrosis.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Min Zhang, Department of Medicine, The Sixth People’s Hospital of Qingdao, 9 Fushun Road, Qingdao 266033, Shandong, China. Tel: +86-53281636667; Fax: +86-53281636688; E-mail: zhangmin150146@126.com

References


miR-122 in liver fibrosis


