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*Original Article*

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

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**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 $CCl_4$. Patients with liver fibrosis had a reduced serum level of miR-122, accompanied with elevated serum ALT and AST levels. Baicalin dose-dependently modulated $CCl_4$-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

## Introducton

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

**Table 1.** Grouping and treatment information

| Group | | Number | Treatment |
|---|---|---|---|
| 1 | Control | 3 | Corn oil i.p., 1% Tween-80 gavage |
| 2 | $CCl_4$ | 5 | 2% $CCl_4$ i.p., 1% Tween-80 gavage |
| 3 | Low dose baicalin + $CCl_4$ | 3 | 2% $CCl_4$ i.p., 100 mg/kg baicalin gavage |
| 4 | Medium dose baicalin + $CCl_4$ | 3 | 2% $CCl_4$ i.p., 200 mg/kg baicalin gavage |
| 5 | High dose baicalin + $CCl_4$ | 3 | 2% $CCl_4$ i.p., 400 mg/kg baicalin gavage |

Notes: $CCl_4$, carbon tetrachloride; i.p., intraperitoneal injection.

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 ($CCl_4$, 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: $CCl_4$ group, low dose baicalin + $CCl_4$ group, medium dose baicalin + $CCl_4$ group, and high dose baicalin + $CCl_4$ 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 20 sec and 60°C 34 sec. The forward primer for miR-122 was TGGAGTGTGACAATGGTGTTTG.

*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: TGACAAGGGTAATGGCGACGC; 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.

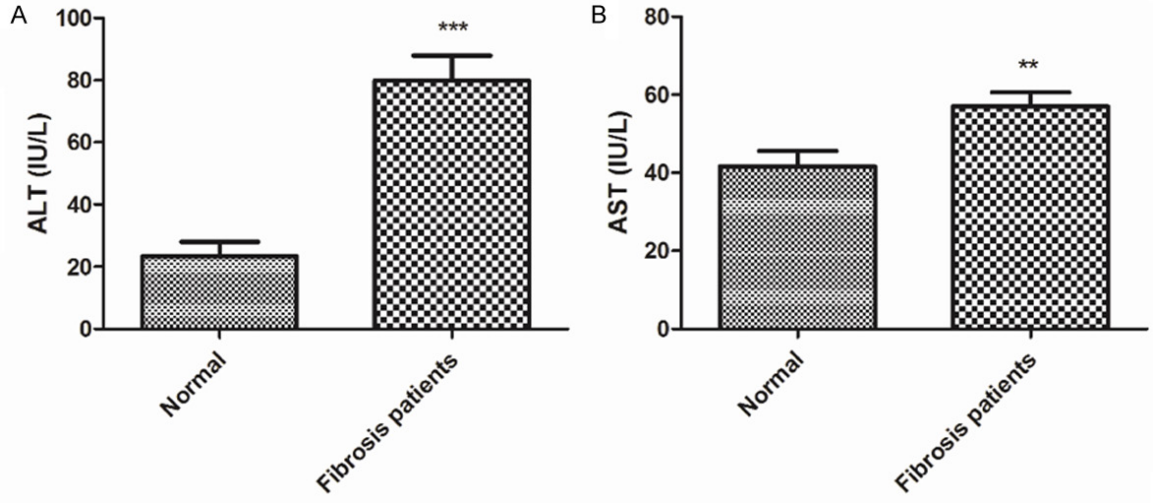


**Figure 1.** AST and ALT levels in healthy controls and liver fibrosis patients. Serum was isolated from healthy controls (Normal) and patients with liver fibrosis, and then used for the detection of ALT (A) and AST (B). ALT, alanine transaminase; AST, aspartate transaminase. **P<0.01, ***P<0.001 vs. normal.

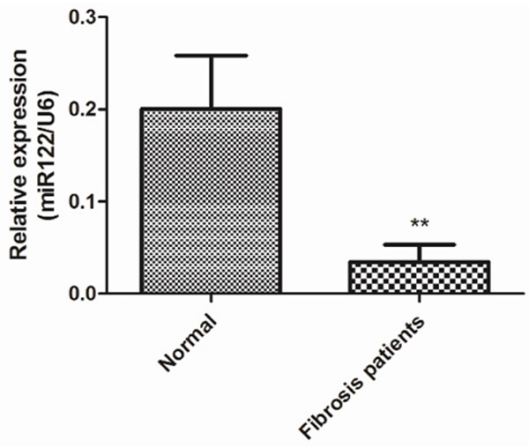


**Figure 2.** Levels in healthy controls and liver fibrosis patients. Serum isolated from liver fibrosis patients and healthy controls was used for the detection of miR-122. U6 snRNA was used as an endogenous control. **P<0.01, ***P<0.001 vs. normal.

*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.

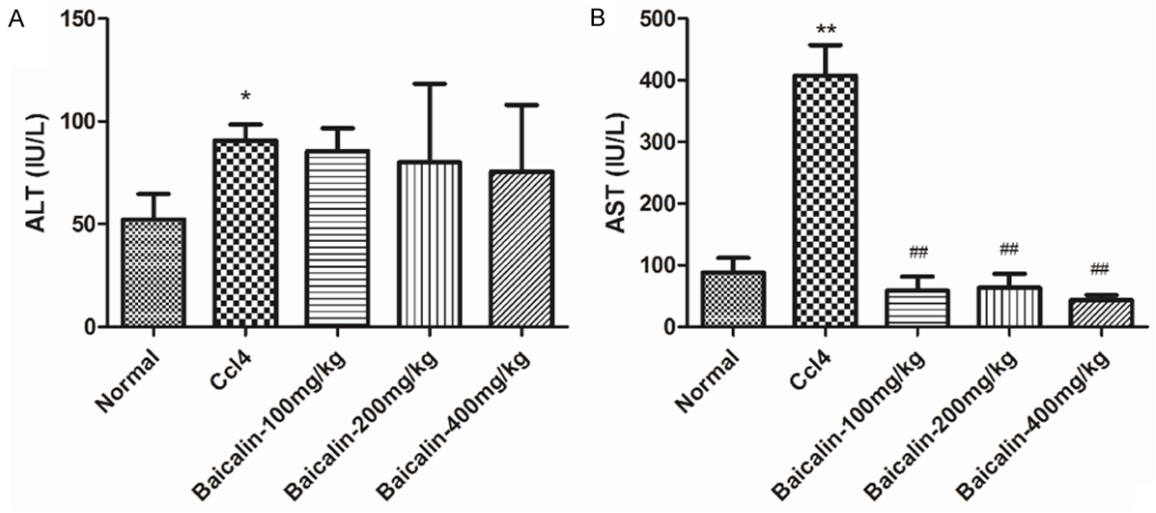


**Figure 3.** Baicalin protected $CCl_4$-induced liver fibrosis in mice. Eight week-old ICR/HaJ mice were treated with 2 mg/kg $CCl_4$, or 2 mg/kg $CCl_4$ + indicated doses of baicalin for 6 weeks to induced liver fibrosis. Serum ALT (A) and AST (B) levels were then measured. ALT, alanine transaminase; AST, aspartate transaminase; $CCl_4$, carbon tetrachloride. *P<0.05, **P<0.01 vs. normal; #P<0.05, ##P<0.01 vs. $CCl_4$.

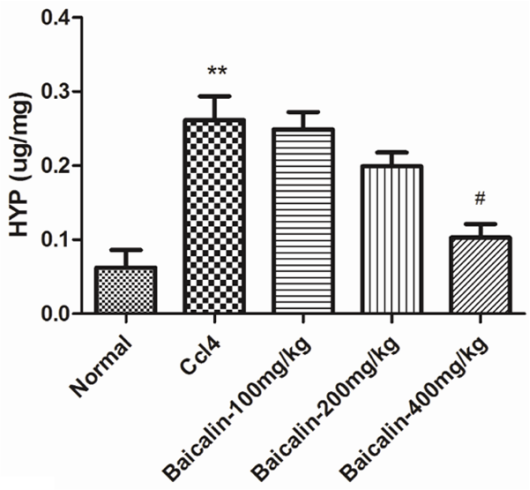


**Figure 4.** Effect of baicalin on hepatic HYP level. Liver was collected for HYP detection after 6 weeks of indicated treatments. $CCl_4$, carbon tetrachloride; HYP, hydrooxyproline. *P<0.05, **P<0.01 vs. normal; #P<0.05 vs. $CCl_4$.

*Baicalin protected $CCl_4$-induced liver fibrosis in mice*

As shown in **Figure 3**, $CCl_4$ 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 hydrooxyproline (HYP) is an important marker for liver fibrosis. We therefore measured HYP content in the liver. Compared to normal group, liver HYP conternt significantly increased in the $CCl_4$-treated mice (P<0.01). Treatment with 400 mg/kg baicalin suppressed $CCl_4$-induced elevation of liver HYP, suggesting the therapeutic effect of baicalin on $CCl_4$-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, $CCl_4$ 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 $CCl_4$ (**Figure 5C-E**). In consistency, the VG staining of liver from control group displayed a normal hepatic lobule structure (**Figure 6A**). Livers from $CCl_4$ group showed disordered hepatocyte tubes and plenty collagen deposition (**Figure 6B**), while baicalin treatment especially the high dose baicalin reduced $CCl_4$-induced collagen deposition (**Figure 6C-E**).

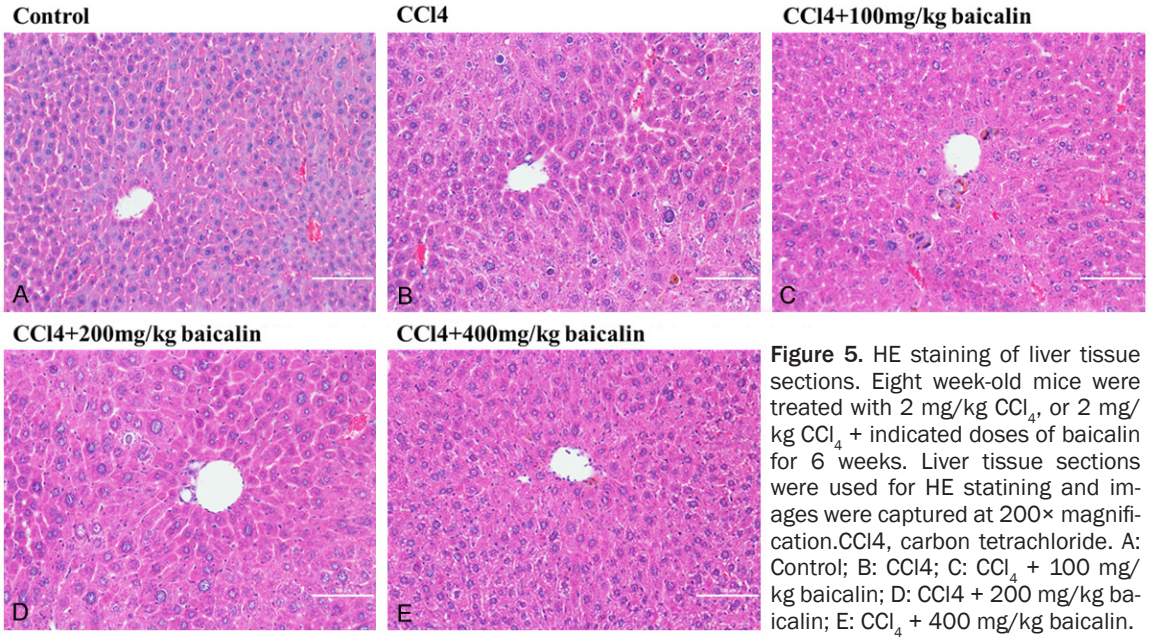


**Figure 5.** HE staining of liver tissue sections. Eight week-old mice were treated with 2 mg/kg $CCl_4$, or 2 mg/kg $CCl_4$ + indicated doses of baicalin for 6 weeks. Liver tissue sections were used for HE statining and images were captured at 200× magnification.CCl4, carbon tetrachloride. A: Control; B: CCl4; C: $CCl_4$ + 100 mg/kg baicalin; D: CCl4 + 200 mg/kg baicalin; E: $CCl_4$ + 400 mg/kg baicalin.

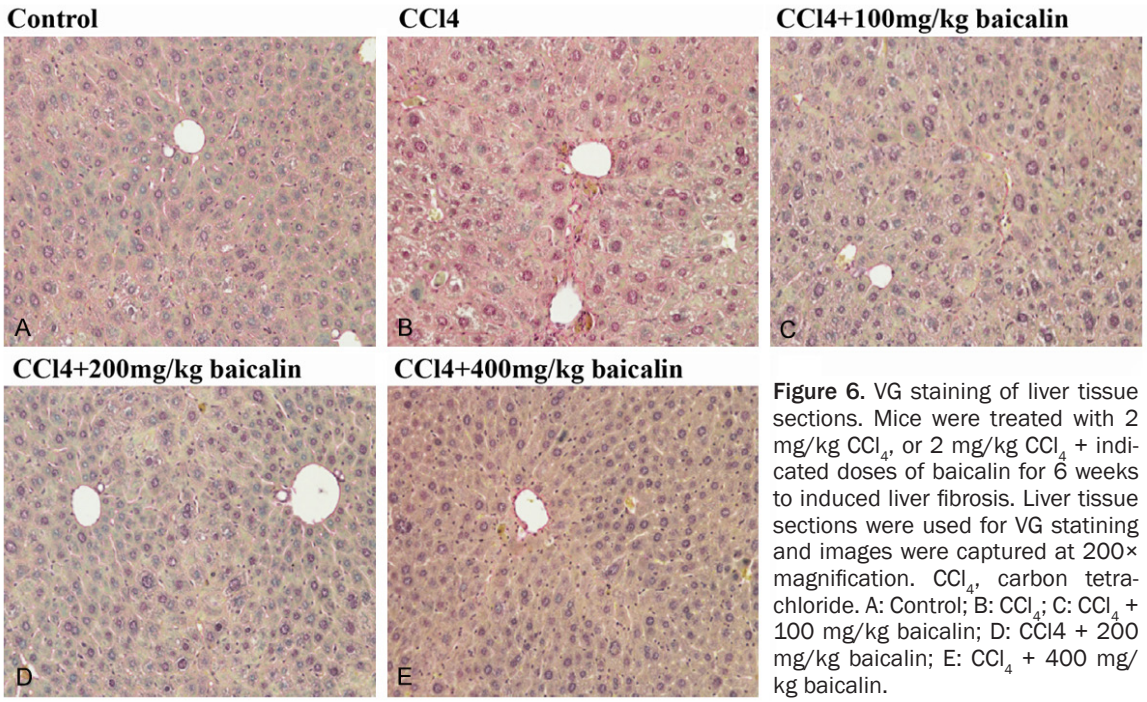


**Figure 6.** VG staining of liver tissue sections. Mice were treated with 2 mg/kg $CCl_4$, or 2 mg/kg $CCl_4$ + indicated doses of baicalin for 6 weeks to induced liver fibrosis. Liver tissue sections were used for VG statining and images were captured at 200× magnification. $CCl_4$, carbon tetrachloride. A: Control; B: $CCl_4$; C: $CCl_4$ + 100 mg/kg baicalin; D: CCl4 + 200 mg/kg baicalin; E: $CCl_4$ + 400 mg/kg baicalin.

*Baicalin increased serum miR-122 in $CCl_4$-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 $CCl_4$-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-

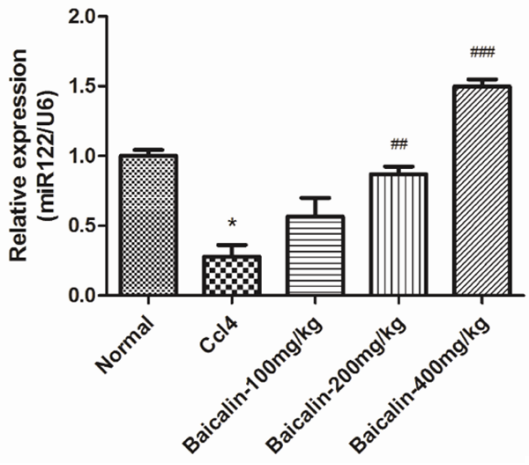


**Figure 7.** Effect of baicalin on serum miR-122 in $CCl_4$-treated mice. Serum isolated from mice treated with 2 mg/kg $CCl_4$, or 2 mg/kg $CCl_4$ + indicated doses of baicalin for 6 weeks was used for the detection of miR-122 expression level. U6 was used as an endogenous control. *P<0.05, **P<0.01 vs. normal; #P<0.05, ##P<0.01, ###P<0.001 vs. $CCl_4$. $CCl_4$, carbon tetrachloride.

tributes to hepatic fibrogenesis [6]. Therefore, we next detected the expression of KLF6 mRNA in the liver. In line with the changes of miR-122, KLF6 increased in $CCl_4$-induced mice and reduced dose-dependently in mice treated with baicalin (**Figure 8B**).

## Discussion

Despite of recent advances in hepatic disorders, current therapies for liver fibrosis are satisfactory and the underlying mechanisms 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 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 $CCl_4$-induced hepatic fibrosis. Baicalin treatment improved liver fibrosis, restored $CCl_4$-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 $CCl_4$-induced liver fibrosis as a model to assess the role of miR-122 in hepatic fibrosis and baicalin-induced therapeutic effect. $CCl_4$ 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. $CCl_4$ 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 $CCl_4$-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 $CCl_4$-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 if miR-122 in the serum and liver of $CCl_4$-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-

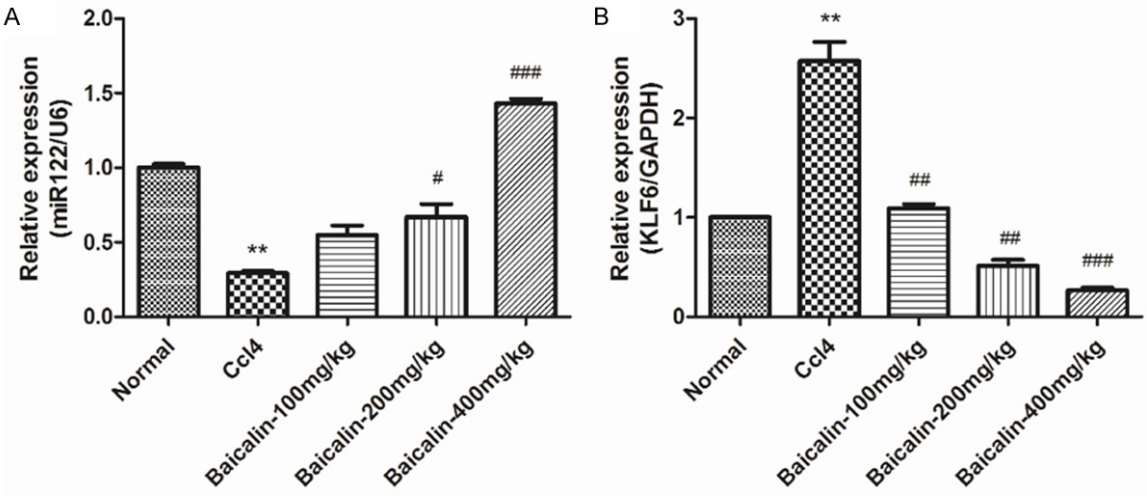


**Figure 8.** Effect of baicalin liver miR-122 and KLF6 mRNA expression in $CCl_4$-treated mice. Liver tissues from mice treated with 2 mg/kg $CCl_4$, or 2 mg/kg $CCl_4$ + indicated doses of baicalin for 6 weeks were detected for miR-122 expression (A) and KLF6 mRNA expression (B). $CCl_4$, carbon tetrachloride; KLF6; krüppel-like factor 6. **P<0.01 vs. normal; #P<0.05, ##P<0.01, ###P<0.001 vs. $CCl_4$.

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.

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