Original Article
Total glucosides of paeony inhibits liver fibrosis and inflammatory response associated with cirrhosis via the FLI1/NLRP3 axis

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Received January 27, 2022; Accepted April 1, 2022; Epub June 15, 2022; Published June 30, 2022

Abstract: Background: Total glucosides of paeony (TGP) has a myriad of hepatoprotective activities. However, its role in cirrhosis, a major risk factor for hepatocellular carcinoma, remains largely unexplored. Here, we determined the impact of TGP on liver fibrosis and inflammation in mice modeled by carbon tetrachloride with an aim to explore a possible molecular mechanism. Methods: Liver fibrosis and inflammation in mice were evaluated using ELISA, hematoxylin-eosin, Masson’s trichrome, immunohistochemical staining and TUNEL methods. The impact of TGP on gene expression in the liver tissues of the mice was investigated using microarray analysis, showing the most significant increase in expression of friend leukemia integration 1 transcription factor (FLI1). After loss-of-functions assays of FLI1, the downstream gene of FLI1 was searched by bioinformatics analysis and verified. Results: TGP reduced liver tissue damage, inhibited apoptosis, and alleviated liver fibrosis and inflammation in cirrhotic mice. FLI1 was downregulated in the liver of cirrhotic mice and lipopolysaccharide-treated hepatocytes, and TGP promoted the expression of FLI1. FLI1 depletion inhibited the effects of TGP on alleviating liver fibrosis and inflammatory responses in mice. FLI1 repressed Nod-like receptor protein 3 (NLRP3) transcription by binding to its promoter. Further silencing of NLRP3 in the presence of shFLI1 alleviated histopathological changes, inhibited apoptosis, and attenuated liver fibrosis and inflammatory responses in the liver of cirrhotic mice. Conclusions: TGP promotes the expression of FLI1, which in turn inhibits NLRP3 expression, thereby reducing cirrhosis-induced liver fibrosis and inflammatory response in mice.

Keywords: Total glucosides of paeony, cirrhosis, FLI1, NLRP3, liver fibrosis

Introduction

Liver cirrhosis is a main driver of morbidity and mortality in developed countries with a one-year mortality rate ranging from 1% to 57% depending on the stage [1]. Cirrhosis develops after chronic inflammation that contributes to replacement of the healthy liver parenchyma with fibrotic tissues and regenerative nodules, leading to portal hypertension [2]. The loss of functional hepatocytes leads to the weakening of liver function, such as the ability to metabolize bilirubin and synthesize proteins [3]. The mechanisms of combating liver fibrosis can be summarized as follows: elimination of injurious factors causing chronic hepatic damage, removal of myofibroblasts, impairment of inflammatory response and activation of anti-inflammatory pathways [4]. Nevertheless, the reversal is often too slow or too sporadic to avoid life-threatening complications, especially in advanced fibrosis [5]. As a consequence, there is an unmet medical need for anti-fibrotic therapies to reverse cirrhosis progression.

Paeoniae Radix Alba, the root of Paeonia lactiflora Pall., has been applied as a traditional medicine for autoimmune diseases related to inflammatory response, and total glucosides of paeony (TGP), the principal active component of Paeoniae Radix Alba, has been verified to show anti-inflammatory and analgesic effects [6]. The alleviating effects of TGP have been well-described in ankylosing spondylitis [7],
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arthriti[8], as well as Sjogren’s syndrome[9]. Single-dose of TGP capsule has been recently found to effectively reduce serum total bile acid in rats with hepatic injury[10]. Also, TGP decreased apoptosis of hepatocytes through the mitochondrial apoptotic pathway in autoimmune hepatitis[11]. In addition, Paeoniflorin (PAE, C23H28O11), the main bioactive components of TGP, exerts protective effect on hepatic fibrosis in rats subjected to radiation[12]. However, the efficacy of TGP on cirrhosis-associated fibrosis and inflammatory responses is less understood. In the present study, we injected carbon tetrachloride (CCl₄) to induce cirrhotic mice[13], followed by microarray analysis of differentially expressed genes between cirrhotic mice and normal mice. It was thus revealed that friend leukemia integration 1 transcription factor (FLI1) was a possible candidate target of TGP in cirrhosis. FLI1, a member of the Ets family, partakes in the development, proliferation, activation and migration of immune cells[14]. Transcription factor FLI1 has been shown to hamper collagen synthesis in cultured dermal fibroblasts[15]. Nevertheless, no conclusive evidence has been found regarding the effects of FLI1 on cirrhosis. Therefore hypothesized that one of the possible mechanisms for the anti-fibrotic, anti-inflammatory and anti-apoptotic properties of TGP in cirrhosis is mediated through the upregulation of FLI1 expression.

Materials and methods

Mice

Sixty-six male C57BL/6 mice (6 weeks old) were acquired from Shanghai Lab Animal Research Center (Shanghai, China). All mice were maintained in a humidity- (55-65%) and temperature-controlled (23 ± 2°C) environment under ad libitum supply of food and water and a 12/12 h day-night cycle. The animal study was ratified by the Ethics Committee of Medical College of Yangzhou University (Approval number: 2021-067-02) and performed following The Guide for the Care and Use of Laboratory Animals published by the US NIH (publication No. 85-23, revised 1996).

CCl₄-induced cirrhosis and treatment

After one week of adaptive feeding, cirrhotic mice (n = 42) were developed by subcutaneous injection of CCl₄ diluted in olive oil (1:1, v/v) two times a week (2 mL/kg) for 6 weeks. The remaining 24 mice were simultaneously injected with equal amount of olive oil and further classified into four groups: the control group (n = 6, no additional treatment), TGP group (n = 6, intragastrical administration of TGP), AAV-NC group (n = 6, tail vein injection of adenovirus AAV-NC), and AAV-shFLI1 group (n = 6, tail vein injection of adenovirus AAV-shFLI1).

The cirrhotic mice were randomly allocated into seven groups: the CCl₄ (n = 6, cirrhotic mouse model), CCl₄ + TGP (n = 6, cirrhotic mice subjected to intragastrical administration of TGP), AAV-NC (n = 6, cirrhotic mice subjected to intragastrical administration of TGP and tail vein injection of adenovirus AAV-NC), AAV-shFLI1 (n = 6, cirrhotic mice subjected to intragastrical administration of TGP and tail vein injection of adenovirus AAV-shFLI1), AAV-NC + AAV-shFLI1 (n = 6, cirrhotic mice subjected to intragastrical administration of TGP and tail vein injection of adenoviruses AAV-shFLI1 and AAV-NC), AAV-NC + AAV-shNLRP3 (n = 6, cirrhotic mice subjected to intragastrical administration of TGP and tail vein injection of adenoviruses AAV-shNLRP3 and AAV-NC) and AAV-shFLI1 + AAV-sh-NLRP3 (n = 6, cirrhotic mice subjected to intragastrical administration of TGP and tail vein injection of adenoviruses AAV-shFLI1 and AAV-sh-NLRP3).

Intragastrical administration of TGP (Ningbo Liwah Pharmaceutical Co., Ltd., Ningbo, Zhejiang, China) at 200 mg/kg (suspended in 0.9% sterile NaCl) once daily for 3 weeks was started at the 4th week of olive oil (containing CCl₄ or not) injection.

AAV-shFLI1, AAV-sh-NLRP3 and empty adenoviral vector AAV-NC were provided by GenePharma (Shanghai, China). Mice requiring adenovirus treatment were injected with 200 μL adenovirus (1 × 10⁹ TU/mL) in the tail vein twice a week for 2 weeks, starting at the 5th week of CCl₄ injection. All mice were euthanized after 6 weeks, and the livers were removed for following assays.

Enzyme-linked immunosorbent assay (ELISA)

Mouse serum was centrifuged at 1500 g for 10 min at 4°C. The levels of liver injury markers ALT (ab282882) and AST (ab263882) in mouse serum were evaluated using ELISA kits (Abcam,
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Cambridge, UK). The concentrations of TNF-α (MTA00B), IL-1β (MLB00C) and IL-6 (M6000B) in the serum of mice were measured by ELISA kits (R&D Systems, Minneapolis, MN, USA).

**HE staining**

Pathological changes in the liver of mice were detected by HE staining as per the instructions of the HE staining kit (Beyotime, Shanghai, China). Mouse liver tissues were fixed with 4% paraformaldehyde, paraffin-embedded, and sliced into 4-μm-thick sections. The above sections were dewaxed with xylene, hydrated with ethanol at gradient concentrations, stained with hematoxylin for 5 min at room temperature, treated with 5% acetic acid for 1 min, and stained with eosin for 1 min. Pathological changes in the liver sections were viewed under a microscope (Olympus Optical Co., Ltd., Tokyo, Japan) after routine dehydration, clearance and sealing.

**Masson’s trichrome staining**

Fibrosis in mouse liver tissues was detected using the Masson’s kit (Servicebio, Wuhan, Hubei, China). Mouse liver tissues were fixed in 4% paraformaldehyde overnight and paraffin-embedded. The 4-μm sections were stained with Masson’s trichrome and examined with Image Pro Plus v7.0 software for fibrosis area analysis. In each section, five random views were captured under a light microscope. The percentage of fibrotic area was measured by comparing the collagen staining area with the total area.

**Immunohistochemistry**

Paraffin-embedded sections of mouse liver tissues were dewaxed with xylene and rehydrated with ethanol at gradient concentrations. Rehydrated tissue sections were incubated in 3% H₂O₂ for 20 min at room temperature and sealed with 100 μL 5% BSA at 37°C for 30 min. The sections were probed with primary antibody against α-SMA (1:1000, ab124964, Abcam) overnight at 4°C and with HRP-labeled secondary goat anti-rabbit antibody against IgG H&L (1:5000, ab205718, Abcam) for 1 h at room temperature. Finally, the sections were treated with DAB (Roche Diagnostics, Indianapolis, IN, USA) and counter-stained with hematoxylin for 30 s before dehydration and fixation.

**TUNEL assay**

Hepatocyte apoptosis was evaluated using the TUNEL Assay Apoptosis Detection Kit (Solarbio, Beijing, China). Liver tissues were sliced into 4-μm paraffin-embedded sections, dewaxed, hydrated, and treated with proteinase K solution for 15 min at room temperature. The sections were cultured with 500 μL TUNEL reaction mixture at 37°C for 1 h and reacted with 100 μL DAB substrate for 10 min. After hematoxylin counter-staining, the sections were sealed with neutral gum. Apoptotic cell nuclei (in yellow-brown or dark brown) were observed under an optical microscope (Eclipse TE2000-U, Nikon Instruments Inc., Melville, NY, USA), and the apoptotic rate was calculated as number of apoptotic nuclei/total nuclei.

**Microarray analysis**

Transcriptome differences in mouse liver due to TGP treatment was analyzed using a mouse GE 4 × 44 K v2 microarray kit (Agilent Technologies, Santa Clara, CA, USA). Total RNA was isolated using a RNeasy mini kit (Qiagen, Valencia, CA, USA), followed by DNase treatment (RNase-Free DNase Set; Qiagen). The total RNA was reversely transcribed into cDNA. After the examination of RNA quality using an Agilent 2100 Bioanalyzer, RNA was labeled using a Low Input Quick Amp Labeling Kit (Agilent) and hybridized using the mouse gene expression microarrays for 17 h at 65°C. Sections were scanned on an Agilent SureScan Microarray Scanner (G2600D) and analyzed using the Feature Extraction Software 11.5.1.1 (Agilent).

**RT-qPCR**

Total RNA was isolated from hepatocytes and liver tissue using TRIzol (Invitrogen, Carlsbad, CA, USA). RNA concentration was measured using a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). RNA was reversely transcribed to cDNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (RR047A, Takara Biotechnology Ltd., Dalian, Liaoning, China). RT-qPCR was car-
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The overexpressed DNA plasmid oe-FLI1 and the control were purchased from GenePharma. After induction of well-grown hepatocytes by lipopolysaccharide (LPS), oe-NC or oe-FLI1 was delivered into the induced cells using the lipfectamine 2000 transfection kit (Thermo Fisher Scientific).

**Dual-luciferase assay**

The potential binding site of FLI1 on the NLRP3 promoter sequence was downloaded from hTFtarget (http://bioinfo.life.hust.edu.cn/hTFtarget/#!/), and NLRP3 promoters containing the binding site (WT) or mutated binding site (MT) were inserted into the pPro-RB-Report vector (RiboBio) to generate a promoter luciferase reporter vector. The above vectors were co-transfected with overexpression of (oe)-NC or oe-FLI1 into NCTC 1469 cells using Lipofectamine 2000, respectively. After 2 d, luciferase assays were carried out using a dual-luciferase reporter gene assay system (Promega, Madison, WI, USA). The firefly luciferase activity was normalized to the Renilla luciferase activity.

**Western blot**

We used radio immunoprecipitation assay lysis buffer (Solarbio) to obtain total protein from hepatocytes and mouse liver tissue. The protein concentrations were assessed using the BCA assay protein assay kit (Solarbio). Proteins (20 μg) were electrophoresed using 10% SDS-polyacrylamide gel and transferred onto PVDF membranes (Millipore Corp., Billerica, MA, USA). Membranes were sealed with 5% skim milk on a shaker for one hour at room temperature and probed with primary antibodies against FLI1 (1:1000, ab133485, Abcam), NLRP3 (1:1000, ab263899, Abcam), and GAPDH (1:10000, ab181602, Abcam) overnight at 4°C. The next day, the membranes were re-probed with secondary antibody goat anti-rabbit IgG H&L (HRP) (1:10,000, ab2057-18, Abcam) for 120 min at room temperature. Immunoreactive bands were measured using an ECL substrate kit (Abcam). Band intensities were quantified and analyzed by ImageJ (NIH, Bethesda, MD, USA).

**Cell transfection**

A mouse normal hepatocyte line NCTC 1469 (CCL-9.1) was purchased from ATCC (Manassas, VA, USA). The cells were cultured in DMEM containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (Thermo Fisher Scientific, MA, USA) at 37°C and 5% CO₂.

**Statistics**

SPSS 22.0 software (IBM Corp., Armonk, NY, USA) was applied for statistical processing. Unpaired t-test was utilized to compare the data between two groups, and one-way ANOVA was used to compare data among multiple groups and followed by Tukey-test. P < 0.05 was accepted as statistically significant. All

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**Table 1. Primers used in RT-qPCR**

<table>
<thead>
<tr>
<th>Name of primer</th>
<th>Sequences (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLI1-F</td>
<td>CCATACAGAAGTCCTCAAGA</td>
</tr>
<tr>
<td>FLI1-R</td>
<td>CATGGTCTGTAGTCCTCAAGG</td>
</tr>
<tr>
<td>NLRP3-F</td>
<td>TCACAACGCACCAAGGGA</td>
</tr>
<tr>
<td>NLRP3-R</td>
<td>AAGAACCAAGCGCAAGCTAG</td>
</tr>
<tr>
<td>GAPDH-F</td>
<td>CATCAGGCACCCAGAAGCT</td>
</tr>
<tr>
<td>GAPDH-R</td>
<td>ATGCCAGTGAGCTCCCGTTCAG</td>
</tr>
</tbody>
</table>

Note: FLI1, friend leukemia integration 1 transcription factor; NLRP3, nod-like receptor protein 3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; F, forward; R, reverse.
numerical data were repeated at least 3 times and expressed as mean ± SD.

Results

TGP attenuates liver fibrosis and inflammatory response in mice

We established a mouse model of cirrhosis by \( \text{CCl}_4 \) treatment. As for control, mice were treated with olive oil. The expressions of ALT and AST, markers of liver injury in mouse serum, were detected by ELISA kits and found to be significantly increased in the \( \text{CCl}_4 \) group. After treatment of TGP, the expressions of ALT and AST in the serum of mice treated with \( \text{CCl}_4 \) were significantly reduced. TGP did not remarkably alter the expression of either in the serum of normal mice (Figure 1A). Subsequently, the liver tissue damage in mice was evaluated by HE staining. The control group had a clear structure of liver lobules, normal structure of the central vein and confluence area; hepatic cell cords arranged radially from the central vein to the surrounding area with irregular hepatic sinusoids, which was not significantly altered by TGP. By contrast, the mice in the \( \text{CCl}_4 \) group showed typical cirrhotic changes with extensive fibrous hyperplasia of liver stroma, more pseudobullets and diffuse inflammatory cell infiltration of interstitial tissue, which were significantly suppressed in the TGP group (Figure 1B). Masson’s staining found that the liver tissue structure of control mice was normal and fibrous deposition was rarely seen, while the liver fibrous tissue of \( \text{CCl}_4 \)-treated mice proliferated significantly and formed wider fibrous spacing. However, the collagen area of liver tissues in mice of the \( \text{CCl}_4 \) + TGP group was significantly reduced, and fibrosis was significantly suppressed (Figure 1C).

We then measured the expression of the fibrosis marker \( \alpha \)-SMA by immunohistochemistry. Relative to the control group, the expression of \( \alpha \)-SMA in the liver tissues of mice in the TGP group did not change significantly. The expression of \( \alpha \)-SMA was much higher in the \( \text{CCl}_4 \) group, which was decreased by TGP treatment (Figure 1D). The results of TUNEL staining demonstrated that there were a small number of apoptotic hepatocytes in the liver tissues of control mice, and TGP showed no significant impact on apoptosis in the control mice. The pro-apoptotic effects of \( \text{CCl}_4 \) on hepatocytes was considerably inhibited after TGP treatment (Figure 1E). Finally, we tested the levels of TNF-\( \alpha \), IL-1\( \beta \) and IL-6 in the serum of mice. It was revealed that the release of TNF-\( \alpha \), IL-1\( \beta \) and IL-6 in the serum of mice was stimulated by \( \text{CCl}_4 \) injections, whereas TGP alleviated the inflammatory response in the serum of cirrhotic mice (Figure 1F). Therefore, TGP treatment alone did not produce significant toxic effects on normal mouse liver, but reduced liver tissue damage, inhibited apoptosis and attenuated liver fibrosis and inflammatory response in cirrhotic mice.

TGP partly reverses the downregulation of transcription factor FLI1 by \( \text{CCl}_4 \)

Differences in gene expression profiles in the liver tissues of cirrhotic mouse resulting from TGP treatment were analyzed by microarray (Figure 2A). We found the most significant difference in FLI1 expression in mouse liver tissues with or without TGP treatment. We then evaluated the expression of FLI1 in mice using RT-qPCR and Western blot. FLI1 was considerably diminished in \( \text{CCl}_4 \)-administrated mice, while FLI1 expression in the liver was partially increased after TGP treatment (Figure 2B, 2C). We then downregulated the FLI1 expression in TGP-treated cirrhotic mice through tail vein injection of AAV-shFLI1 or AAV-NC. The result of RT-qPCR and Western blot results revealed that AAV-NC did not significantly affect FLI1 expression in the liver of TGP-treated cirrhotic mice, while AAV-shFLI1 significantly inhibited FLI1 expression in mouse liver tissues (Figure 2D, 2E).

FLI1 inhibits the effects of TGP on alleviating liver fibrosis and inflammatory response in mice

The levels of ALT and AST were considerably augmented in mouse serum after inhibition of FLI1 by ELISA analysis (Figure 3A). The results of HE staining exhibited that mice in the AAV-shFLI1 group had increased liver pathology, enhanced pseudobullets and augmented inflammatory cell infiltration (Figure 3B). By Masson’s staining, we observed that inhibition of FLI1 aggravated liver fibrosis in mice with a significant promotion in the collagen area of liver tissues (Figure 3C). Similar results were obtained by immunohistochemical analysis of the fibrosis marker \( \alpha \)-SMA, and inhibition of FLI1 increased the expression of \( \alpha \)-SMA (Figure 3D).
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Figure 1. TGP attenuates cirrhosis-induced liver fibrosis and inflammation in mice. A. Detection of ALT and AST levels in serum of mice with cirrhosis by ELISA. B. The extent of liver tissue damage in cirrhotic mice assessed using HE staining. C. Fibrosis in the liver of cirrhotic mice measured using Masson’s staining. D. Immunohistochemical analysis of α-SMA positivity in liver tissues of cirrhotic mice. E. Apoptosis in mice with liver cirrhosis measured using TUNEL assay. F. The determination of TNF-α, IL-1β and IL-6 in the serum of mice with cirrhosis examined using ELISA assay. Data are displayed as the mean ± SD (n = 6) and compared by one-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001. TGP, total glucosides of paeony; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ELISA, enzyme-linked immunosorbent assay; HE, hematoxylin-eosin; α-SMA, alpha skeletal muscle actin; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated 2’-Deoxyuridine 5’-Triphosphate (dUTP) nick end labeling; TNF-α, tumor necrosis factor alpha; IL, interleukin.

TUNEL results showed that the apoptosis was significantly enhanced in the AAV-shFLI1-administered mice (Figure 3E). Finally, ELISA analysis revealed a significant elevation in the
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Figure 2. TGP can promote the expression of transcription factor FLI1. (A) Microarray analysis of differential gene expression profiles in the liver of mice treated with CCl₄ or CCl₄ + TGP. (B, C) Detection of FLI1 expression in liver tissues of mice by RT-qPCR (B) and Western blot (B) and Western blot (C) and Western blot (C). (D, E) Detection of FLI1 mRNA and protein expression after tail vein injection of AAV-shFLI1 or AAV-NC in CCl₄ + TGP-treated mice by RT-qPCR (D) and Western blot (D) and Western blot (E). Data are displayed as the mean ± SD (n = 6) and compared by one-way ANOVA. *P < 0.05, **P < 0.01. TGP, total glucosides of paeony; FLI1, friend leukemia integration 1 transcription factor; CCl₄, carbon tetrachloride; AAV, adeno-associated virus; NC, negative control.

is a significant rise in NLRP3 expression in the liver of cirrhotic mice, a significant decline in the level of NLRP3 after TGP treat-
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A

ALT (μg/mL)

AAV-NC  AAV-shFLI1

300

200

100

0

AAV-NC  AAV-shFLI1

400

300

200

100

0

**

**

B

AAV-NC  AAV-shFLI1

**

**

C

Relative Fibrosis Area (%)

AAV-NC  AAV-shFLI1

25

20

15

10

5

0

AAV-NC  AAV-shFLI1

**

**

D

α-SMA Positive Rate (%)

AAV-NC  AAV-shFLI1

25

20

15

10

5

0

AAV-NC  AAV-shFLI1

*

E

Apoptosis Rate (%)

AAV-NC  AAV-shFLI1

15

10

5

0

AAV-NC  AAV-shFLI1

**

**

F

TNF-α (pg/mL)

IL-1β (pg/mL)

IL-6 (pg/mL)

AAV-NC  AAV-shFLI1

AAV-NC  AAV-shFLI1

AAV-NC  AAV-shFLI1

*

*

**

**

G

FLI1

GAPDH

Control  AAV-NC  AAV-shFLI1

51 kDa

36 kDa

Relative FLI1 Protein Expression

AAV-NC  AAV-shFLI1

**

**

H

Control  AAV-NC  AAV-shFLI1
Figure 3. AAV-shFLI1 inhibits the effects of TGP on liver fibrosis and inflammatory responses associated with cirrhosis in mice. Adenoviruses AAV-shFLI1 or AAV-NC were injected into CCl₄ + TGP-treated mice via tail vein injection. A. The serum levels of ALT and AST measured using ELISA in mice. B. The extent of liver tissue damage in CCl₄ + TGP-treated mice assessed using HE staining. C. Fibrosis in the liver of CCl₄ + TGP-treated mice measured using Masson’s staining. D. Immunohistochemical analysis of α-SMA positivity in liver tissues of CCl₄ + TGP-treated mice. E. Apoptosis in CCl₄ + TGP-treated mice measured using TUNEL assay. F. The determination of TNF-α, IL-1β and IL-6 in the serum of CCl₄ + TGP-treated mice examined using ELISA assay. Adenoviruses AAV-shFLI1 or AAV-NC were injected into olive oil-treated mice via tail vein injection. G. Detection of FLI1 protein expression in liver tissues of olive oil-treated mice by Western blot (original, full-length gel and blot images can be found in the Supplementary Figure 1). H. The extent of liver tissue damage in olive oil-treated mice assessed using HE staining. I. Fibrosis in the liver of olive oil-treated mice measured using Masson’s staining. J. Immunohistochemical analysis of α-SMA positivity in liver tissues of olive oil-treated mice. Data are displayed as the mean ± SD (n = 6) and compared by one-way ANOVA or unpaired t test. *P < 0.05, **P < 0.01. FLI1, friend leukemia integration 1 transcription factor; CCl₄, carbon tetrachloride; AAV, adeno-associated virus; NC, negative control; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ELISA, enzyme-linked immunosorbent assay; HE, hematoxylin-eosin; α-SMA, alpha skeletal muscle actin; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated 2’-Deoxyuridine 5’-Triphosphate (dUTP) nick end labeling; TNF-α, tumor necrosis factor alpha; IL, interleukin.
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Figure 4. FLI1 represses NLRP3 transcription. (A) Pathway enrichment analysis of downstream targets of FLI1. (B) Protein-protein interactions (PPI) enrichment analysis of downstream targets of FLI1. (C) FLI1 has a possible binding relation with the NLRP3 promoter region. (D, E) Expression of NLRP3 in the liver of mice with cirrhosis by RT-qPCR (D) and Western blot (E) (original, full-length gel and blot images can be found in the Supplementary Figure 1). (F, G) Expression of FLI1 and NLRP3 in LPS-treated mouse hepatocytes assessed by RT-qPCR (F) and Western blot (G) (original, full-length gel and blot images can be found in the Supplementary Figure 1). (H, I) Effects of transfection with oe-FLI1 on FLI1 and NLRP3 expression in LPS-treated hepatocytes by RT-qPCR (H) and Western blot (I) (original, full-length gel and blot images can be found in the Supplementary Figure 1). (J) Binding site of FLI1 on the NLRP3 promoter. (K) The effect of FLI1 on NLRP3 transcription evaluated using the luciferase reporter assay. (L) The binding relation between FLI1 and NLRP3 promoter measured using ChIP-qPCR. Data are displayed as the mean ± SD of three independent experiments (n = 6) and analyzed by unpaired t test or one-way/two-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001. FLI1, friend leukemia integration 1 transcription factor; NLRP3, nod-like receptor protein 3; LPS, lipopolysaccharide; ChIP, chromatin immunoprecipitation.
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To examine the role of NLRP3 on liver fibrosis and inflammation in mice, we injected TGP-treated cirrhotic mice with AAV-NC + AAV-shNLRP3 or AAV-shFLI1 + AAV-shNLRP3. Western blot analysis showed a significant decline in NLRP3 protein expression upon silencing of NLRP3, but no significant change in FLI1 expression (Figure 4A). The results of ELISA showed that AAV-shNLRP3 significantly attenuated the inhibitory effect of AAV-shFLI1 on TGP efficacy, whereas inhibition of NLRP3 alone significantly induced the repressive effects of TGP on the levels of ALT and AST (Figure 5B). By HE staining, we observed that inhibition of NLRP3 reversed the accentuation in liver damage caused by AAV-shFLI1 and significantly ameliorated the pathological structural changes in the liver of cirrhotic mice (Figure 5C). Meanwhile, it was shown by Masson’s staining and immunohistochemical assay that knockdown of NLRP3 inhibited liver fibrosis and decreased α-SMA expression in the liver tissues, which were more pronounced in the livers of mice not treated with AAV-shFLI1 (Figure 5D, 5E). TUNEL staining showed that the apoptosis of murine hepatocytes was significantly reduced after AAV-shNLRP3 injection and the hepatocyte-protective effects of TGP was promoted (Figure 5F). Finally, ELISA analysis revealed a substantial decrease in serum levels of TNF-α, IL-1β and IL-6 in mice after further inhibition of NLRP3 (Figure 5G). In summary, further silencing of NLRP3 in TGP-treated cirrhotic mice with FLI1 inhibition alleviated histopathological changes in the liver, inhibited apoptosis, and attenuated liver fibrosis and inflammatory responses. Moreover, inhibition of NLRP3 alone further promoted the therapeutic effect of TGP on cirrhotic mice.

Discussion

TGP is comprised of paeoniflorin, hydroxyl-paeoniflorin, paeonin, albiflorin and benzoylpaeoniflorin [16]. The anti-inflammatory, anti-oxidative, and anti-hepatic injury properties of TGP have been proven to be without evident toxic or side effects [17]. In this study, the role of TGP in liver fibrosis and inflammatory responses in the treatment of cirrhosis was investigated. Deletion of FLI1 in mice enhanced cell apoptosis, liver fibrosis and inflammatory responses. Furthermore, mechanistic studies found that FLI1 repressed NLRP3 transcription. Thus, TGP might represent a possible candidate for the treatment of cirrhosis.

TGP has been evidenced to ameliorate pristane-induced lupus nephritis [18], restore intestinal barrier function [19] and delay onset of Sjogren’s syndrome [20]. In this study, we found that TGP was effective in repressing liver injury markers (ALT and AST) and pro-inflammatory factors (TNF-α, IL-1β and IL-6) in the serum, and in reducing TUNEL-positive cells, collagen area and α-SMA-positivity in the liver tissues. Similarly, TGP has the potential to be a treatment approach for diabetic liver injury by diminishing liver lipid accumulation and inflammatory response [21]. More specifically, the administration of TGP protected the rats from CCl₄-induced acute liver injury and ALT and AST elevation, in addition to hepatocyte apoptosis and inflammation [22]. The anti-inflammatory effects of TGP have also been validated in Sjögren’s syndrome [23], gouty arthritis [24], and kidney ischemia/reperfusion injury [25]. TGP increased viability and hampered apoptosis of hypoxia/reoxygenation-treated renal cells [26]. The tubulointerstitial injury was alleviated and α-SMA expression was reduced in diabetic rats administered orally with TGP [27].
Figure 5. Silencing of NLRP3 mitigates liver fibrosis and inflammatory response in mice treated with AAV-shFLI1 and TGP. TGP-treated mice were injected with AAV-NC + AAV-shFLI1, AAV-NC + AAV-shNLRP3, AAV-shFLI1 + AAV-shNLRP3 via tail vein injection. A. Detection of FLI1 and NLRP3 protein expression in liver tissues of mice (original, full-length gel and blot images can be found in the Supplementary Figure 1) using Western blot. B. The serum levels of ALT and AST were assessed by ELISA in mice. C. The extent of liver tissue damage in mice assessed using HE staining. D. Fibrosis in the liver of mice measured using Masson’s staining. E. Immunohistochemical analysis of α-SMA positivity in liver tissues of mice. F. Apoptosis in mice with liver cirrhosis measured using TUNEL assay. G. The determination of TNF-α, IL-1β and IL-6 in the serum of mice examined using ELISA assay. Data are displayed as the mean ± SD (n = 6) and analyzed by one-way/two-way ANOVA. *P < 0.05, **P < 0.01. FLI1, friend leukemia integra-
Subsequently, we set to decipher the mechanism underlying TGP in cirrhosis. Microarray analysis showed that FLI1 might be the reason for the anti-inflammatory and anti-fibrotic properties of TGP in cirrhosis. Chan et al. revealed that FLI1 was a vital mediator of the fibrogenic actions of adenosine in scleroderma [28]. Bujor et al. also showed that FLI1 downregulation in scleroderma myeloid cells elicited pro-fibrotic and pro-inflammatory effects [29]. Interestingly, motif analysis of the upstream genomic sequences of hepatocellular carcinoma-associated nonalcoholic fatty liver disease gene module revealed that the enriched motifs were bound by the transcription factor FLI1 [30], indicating the possible interaction of FLI1 and liver diseases. Here, we observed that the loss of FLI1 using AAV-shFLI1 sufficiently repressed the alleviating effects of TGP on liver fibrosis and inflammatory response. In the transcriptome-wide analysis of transcriptional regulators of sensome and inflammation genes in retinal microglia conducted by Saddala et al., FLI1 was revealed as one of the top 10 transcription factors that are differentially expressed between the TNFα/IFNγ-activated and the non-activated microglia [31]. It was thus suggested that the effects of FLI1 on cirrhosis was mediated by its role as a transcription factor.

Integrated bioinformatics prediction showed that NLRP3 is a putative target modulated by FLI1. NLRP3 has been implicated in the pathogenesis of alcohol-associated liver disease, nonalcoholic fatty liver disease/nonalcoholic steatohepatitis, as well as fibrosis [32]. MyD88 in macrophages enhanced liver fibrosis by activating the NLRP3 inflammasome in hepatic stellate cells [33]. As for its individual role, NLRP3 was found increased in renal tubular epitheliums from biopsies of patients with chronic kidney disease, and persistent NLRP3 overexpression was related to chronic pathological changes following acute kidney injury [34]. In addition to its well-known pro-inflammatory effects, exposure to hyperandrogen drives ovarian dysfunction and fibrosis by inducing NLRP3 in mice as well [35]. Knockdown of NLRP3 was revealed here to rescue the effects of AAV-shFLI1 in vivo. Also, liver damage induced by bile acid and CCl4 injection was ameliorated in NLRP3−/− mice, as evidenced by reduced Sirius red-stained areas and a decrease in mRNA expression of α-SMA [36, 37].

Here, we concentrated on the role of TGP in liver fibrosis and inflammatory responses in a mouse model of cirrhosis. Our studies specifically proved that: (1) TGP alleviates cell apoptosis, liver fibrosis and inflammatory responses in mice; (2) TGP exerts its function in cirrhosis by promoting the expression of FLI1; (3) knockout of FLI1 accentuates cirrhosis in mice in the presence of TGP; (4) FLI1 modulates the transcription of NLRP3; (5) knockout of NLRP3 further ameliorates the effects of shFLI1 on mice. There are also limitations in this study, such as the absence of results regarding the phenotype of NCTC 1469 cells in vitro after gene expression alteration, so additional studies are needed.

Disclosure of conflict of interest

None.

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References


TGP attenuates liver fibrosis and inflammation


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Supplementary Figure 1. Original western blots.