Application of TGF-β1, TIMP-1 and TIMP-2 small interfering RNAs can alleviate CCl_4-induced hepatic fibrosis in rats by rebalancing Th1/Th2 cytokines

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Received September 14, 2020; Accepted May 17, 2021

DOI: 10.3892/etm.2021.10395

Abstract. The present study aimed to investigate the effects of TGF-β1, tissue inhibitor of metalloproteinase (TIMP)-1 small interfering (si)RNA and TIMP-2 siRNA on hepatic fibrosis in rats and explore the T helper (Th)1/Th2 balance. Moreover, IFN-γ, IL-4 and IL-13 are the main cytokines associated with Th1/Th2 responses and have significant influence on the progression of hepatic fibrosis. The expression levels of IFN-γ, IL-4 and IL-13 in rats with hepatic fibrosis that were treated with siRNAs against the aforementioned molecules were measured using various techniques including immunohistochemical staining, western blotting and reverse transcription-quantitative PCR. The principal outcomes revealed the downregulation of IFN-γ and the upregulation of IL-4 and IL-13 in the model group compared with the normal group. Moreover, the expression of IFN-γ was significantly increased, while IL-4 and IL-13 demonstrated no significant difference in the TGF-β1 siRNA treatment group compared with the model group. The TIMP-1 and TIMP-2 siRNA treatment groups exhibited significantly increased expression levels of IFN-γ, but lower expression levels of IL-4 and IL-13 compared with the model group. These results indicated that TIMP-1 and TIMP-2 were improved antifibrotic targets compared with TGF-β1.

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Abbreviations: TIMP, tissue inhibitor of metalloproteinase; HSC, hepatic stellate cell; ECM, extracellular matrix

Key words: hepatic fibrosis, small interfering RNA, T helper 1, T helper 2, interleukin-4, interleukin-13, interferon-γ

Introduction

In the occurrence and development of various chronic liver diseases, such as chronic viral hepatitis, alcoholic hepatitis and drug induced hepatitis, liver fibrosis is a notable indicator of pathological stage (1). The mechanism of liver fibrosis involves the imbalance of synthesis and degradation of the extracellular matrix (ECM), and the central feature is the activation and proliferation of hepatic stellate cells (HSCs) (1). The activation of HSCs is mediated by various cytokines, such as TGF-β1, matrix metalloproteinases and connective tissue growth factor (2). T helper (Th)1/Th2 immune imbalance has been indicated to contribute to fibroblast activation, proliferation and transformation to HSCs, leading to increased ECM production (3,4). Our previous studies have confirmed that small interfering (si)RNA can successfully interfere with the expression levels of TGF-β1, TIMP-1 and TIMP-2 in rat liver tissue. The results indicated that in the TGF-β1, TIMP-1 and TIMP-2 siRNA treatment groups, TGF-β1, TIMP-1 and TIMP-2 proteins expression levels were significantly decreased compared with those in the negative control groups (5,6). Therefore, the expression levels of Th1 (IFN-γ) and Th2 (IL-4 and IL-13) cytokines were investigated to explore ways to improve hepatic fibrosis in rats with siRNA treatment under similar experimental conditions.

Materials and methods

Materials and study design. The hepatic tissue was obtained from our previous studies. Healthy male Sprague-Dawley rats (6-weeks-old) weighing 160-210 g were provided by the Laboratory Animal Center of Chongqing Medical University. A total of 60 rats were randomly divided into six groups (10 rats per group): Normal, model, negative control siRNA, TGF-β1 siRNA, TIMP-1 siRNA and TIMP-2 siRNA groups. The rat fibrosis model was established using CCl_4 combined with a high fat and high cholesterol diet. Except for the normal group, all the rats were subcutaneously injected with 40% CCl_4 (ratio, CCl_4: Liquid paraffin; 2:3). The first dose was 3 ml/kg, followed by a dose of 2 ml/kg injected twice a week for 12 weeks. In the second week, the treatment groups and

the negative control group received a 0.25 mg/kg dose of the corresponding viral plasmid which was constructed from previous experiments (7). The injection volume was 5 ml/kg, the normal and model groups received the same volume of a 0.9% sodium chloride solution. All groups received these injections via the tail vein twice a week for 12 weeks. The normal group was administered a normal diet, while the other groups were fed with a diet high in fat and cholesterol. All rats had free access to food and water *ad libitum*. All rats were kept at 25˚C under 12 h dark/light cycles inside the air-conditioned room with ~70% humidity. All rats were sacrificed after 12 weeks. The liver tissues from the middle left lobe of each liver were stored in 4% formalin and embedded in paraffin; a portion of the liver tissue samples were stored in liquid nitrogen in a -80˚C refrigerator for further analysis. All animal experimental operations were in accordance with Chongqing Management Approach of Laboratory Animals (Chongqing Government order no. 195). The animals from the present study received humane care that was approved by the Institutional Animal Care and Ethics Committee of the Second Affiliated Hospital of Chongqing Medical University (Chongqing, China). Additionally, our previous study demonstrated the results of a comprehensive analysis of H&E and Sirius red staining as follows: i) In the normal group, the structure of the hepatic lobule was normal and fibrosis was grade 0; ii) in the model group and the negative control group, a typical pseudo-lobular structure was formed and fibrosis was grade V-VI; iii) compared with the model group and negative control group, the damage of hepatic lobules in the treatment group was significantly reduced, and the majority of the pathological grades were II-VI (Fig. 1) (5).

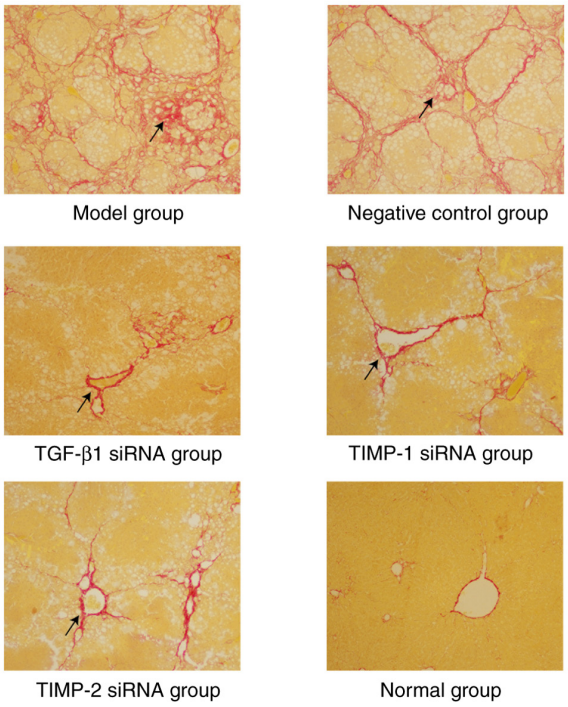

Figure 1. Sirius red staining of rat liver tissue (magnification, x100). TIMP, tissue inhibitor of metalloproteinase; si, small interfering.

Immunohistochemical staining. Paraffin-embedded sections were deparaffinized with xylene, sliced to a thickness of 3-5 μm, and dehydrated in decreasing concentrations of alcohol. The tissue sections were incubated with 3% hydrogen peroxide for 5-10 min to block endogenous peroxidase activity and 5-10% normal goat serum (OriGene Technologies, Inc.) was used to block non-specific binding sites at room temperature for 20 min. The sections were incubated at 4˚C overnight with rabbit polyclonal antibodies against bioactive IFN-γ (1:100; cat. no. bs-0480R, BIOSS), IL-4 (1:200; cat. no. bs-0581R, BIOSS) and IL-13 (1:200; cat. no. bs-0560R, BIOSS). Unbound antibody was washed off with PBS, and the slides were incubated with the secondary antibody biotin-labeled sheep anti-rabbit IgG (1:100; cat. no. SA1096; Wuhan Boster Biological Technology, Ltd.) at 37˚C for 20 min. The antigen-antibody complexes were detected by the Type I SABC immunohistochemical kit (cat. no. SA1094, Wuhan Boster Biological Technology, Ltd.) for immunostaining visualization. All the above procedures were performed in accordance with the manufacturer's protocol. The staining intensities of IFN-γ, IL-4 and IL-13 were quantified using Image-Pro Plus software 6.0 (Media Cybernetics, Inc.).

Western blotting. Frozen hepatic tissues were mixed well with ice-cold buffer (50 mM pH 8.0 Tris; 5 mM EDTA; 150 mM NaCl; 0.5% Nonidet P-40; 100 mM PMSF; 1 mg/ml leupeptin; 1 mg/ml aprotinin; and 1 M DTT) for 30 min on ice, the samples were centrifuged at 12,000 x g at 4˚C for 5 min and the supernatant was collected. Protein concentration was determined using the BCA method. Subsequently, 50 μg of total protein per lane was resolved on 15% SDS-PAGE and transferred to PVDF membranes (MilliporeSigma), which were blocked with 5% BSA (Beijing Solarbio Science & Technology Co., Ltd.) at room temperature for 1 h while shaking. The membranes were incubated overnight at 4˚C with primary antibodies [IFN-γ (1:100; cat. no. bs-0480R, BIOSS), IL-4 (1:200; cat. no. bs-0581R, BIOSS), IL-13 (1:200; cat. no. bs-0560R, BIOSS) and β-actin (1:400; cat. no. BM387; Wuhan Boster Biological Technology, Ltd.)] while shaking, washed three times with 0.05% TBS-Tween-20 and then incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:2,000; cat. no. BM2006; Wuhan Boster Biological Technology, Ltd.) for 1 h with shaking at room temperature. Finally, chemiluminescence (DAB kit; cat. no. SA2025; Wuhan Boster Biological Technology, Ltd.) was used to detect the expression levels of IFN-γ, IL-4, IL-13 and β-actin. The intensity of the protein bands was measured using Bandscan version 5.0 (Bio-Rad Laboratories, Inc.) and β-actin was used as the internal control.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated with a high purity total RNA rapid extraction kit (cat. no. RP1202; BioTeke Corporation), and then the concentration was determined with electrophoresis and a UV spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). Total RNA was converted into cDNA at 37˚C for 15 min followed by 85˚C for 5 sec using the ExScript™ RT reagent kit (cat. no. RR037B; Takara Biotechnology Co., Ltd.). RT-qPCR was performed on an ABI Prism 7300 PCR instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.) using SYBR® Green as the detection fluorophore. Each

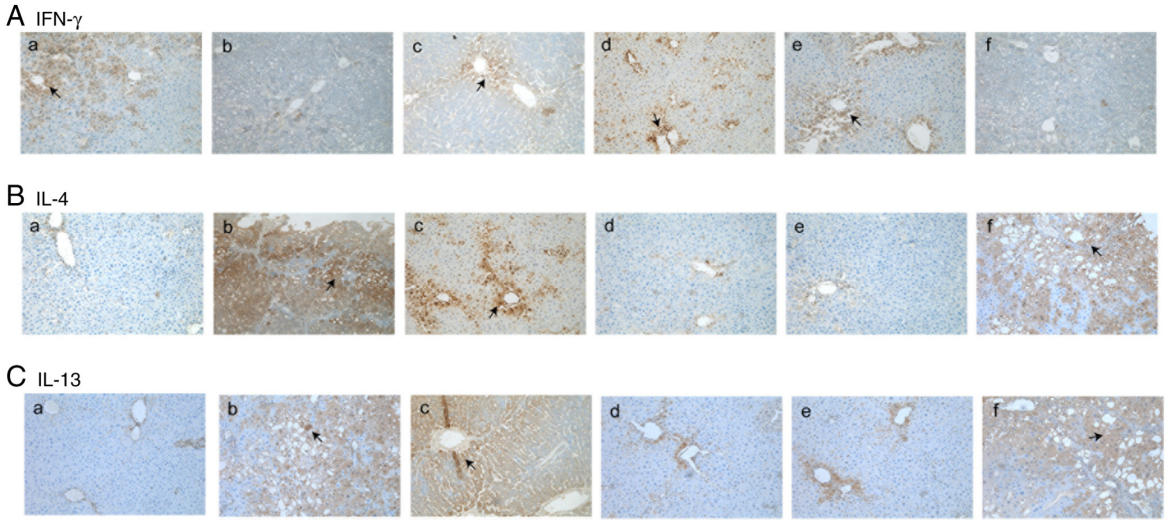

Figure 2. Immunohistochemistry of liver tissue. (A) INF-γ, (B) IL-4, (C) IL-13. (a) Normal group, (b) model group, (c) TGF-β1 siRNA treatment group, (d) TIMP-1 siRNA treatment group, (e) TIMP-2 siRNA treatment group and (f) negative control group (magnification, x200). TIMP, tissue inhibitor of metalloproteinase; si, small interfering.

20 μl reaction mixture contained 2 μl cDNA, 10 μl 2x SYBR® Premix Ex Taq (Takara Biotechnology Co., Ltd.), 1.6 μl of 10 μmol/μl forward and reverse primers and ddH2O to final volume. Optimization was performed for each gene-specific primer prior to the experiment to confirm that the primer concentrations and reaction conditions did not produce artificial amplification signals. Primers were designed using the IFN-γ, IL-4 and IL-13 sequences provided by GenBank (https://www.ncbi.nlm.nih.gov/genbank/), and the endogenous GAPDH gene was used as a control. The sequences of the primers were as follows: IFN-γ forward, 5'-GCGTCCCAAGAAGCAGAATGA-3' and reverse, 5'-TCCGTGTGGACGAATCATCA-3'; IL-4 forward, 5'-ACTCCATGCACCGAGATGTTT-3' and reverse, 5'-CTGGAAGCCCTGCAGATGAG-3'; IL-13 forward, 5'-CTGAGCAACATCACACAAG-3' and reverse, 5'-GGTTACAGAGGCCATTCAAT-3'; and GAPDH forward, 5'-TGATTCTACCCACGGCAAGTT-3' and reverse, 5'-TGATGGGTTTCCCATTGATGA-3'. A standard two-step PCR amplification procedure was used. The PCR parameters were as follows: 95˚C for 10 sec, followed by 40 cycles of 5 sec at 95˚C and 31 sec at 60˚C. RT-qPCR was performed at least three times per gene, with a no-template control as a negative control. The relative mRNA levels of the target genes were calculated using the $2^{-\Delta\Delta Cq}$ method (8).

Statistical analysis. Each experiment was repeated at least three times, and the experimental data are presented as the mean ± standard deviation. Statistical analysis was performed using SPSS (version 13.0; SPSS, Inc.). Differences between groups were analyzed using one-way ANOVA followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Immunohistochemical characteristics of each group of rats. The expression level of IFN-γ in the normal group was mainly localized in the portal area and the area surrounding the central vein. Compared with that of the normal group, the expression level of IFN-γ in the model group was significantly decreased (Fig. 2A-a and b; Table I). The expression levels of IFN-γ in the TGF-β1, TIMP-1 and TIMP-2 siRNA treatment groups were significantly increased compared with that in the model group (Fig. 2A-b-e). In addition, there was no notable difference in the expression levels of IFN-γ between the negative control group and the model group (Fig. 2A-b and f; Table I).

The normal group presented low expression levels of IL-4 and IL-13 (brown particles in the cytoplasm; Fig. 2B-a and C-a). These cytokines were mainly concentrated in the area surrounding the central vein and in the portal area. The expression levels of IL-4 and IL-13 in the model group were significantly increased compared with those of the normal group, as indicated by immunohistochemical staining (Fig. 2B-b and C-b; Table I). It was observed that the expression levels of IL-4 and IL-13 in the TGF-β1 siRNA treatment group were similar to those in the model group (Fig. 2B-c and C-c); however, compared with the model and negative control group, the expression levels of IL-4 and IL-13 in the TIMP-1 and TIMP-2 siRNA treatment groups were significantly decreased (Fig. 2B and C-d and e; Table I).

Expression levels of IFN-γ, IL-4 and IL-13 as determined by western blotting. Western blotting was used to examine the protein expression levels in each group. Analysis of the gray values of the bands revealed that the β-actin bands were uniform, but the densities of the target gene bands were different (Fig. 3). Compared with those of the normal group, the expression level of IFN-γ in the model group was decreased, but the levels of IL-4 and IL-13 were increased. This effect was the same in the TGF-β1 siRNA treatment group (group A) and the TIMP-1 and TIMP-2 siRNA treatment groups (group B). In the TGF-β1, TIMP-1 and TIMP-2 siRNA treatment groups, the expression level of IFN-γ was significantly increased compared with that of the model group (Table II). The expression levels of IL-4 and IL-13 in the TGF-β1 siRNA treatment group were similar to those in the model group, while the expression levels of IL-4

Table I. Expression of IFN-γ, IL-4 and IL-13 detected via immunohistochemistry.

Cytokine	Normal group	Model group	TGF-β1 siRNA treatment group	TIMP-1 siRNA treatment group	TIMP-2 siRNA treatment group	Negative control group
IFN-γ	13.035±1.517	2.931±0.903[a]	8.178±0.562[b]	9.289±1.026[b]	9.534±0.627[b]	2.752±0.045[a]
IL-4	5.482±1.313	16.737±2.011[a]	14.787±1.068[c]	7.486±0.448[b]	7.513±1.152[b]	15.260±1.205[a]
IL-13	4.457±0.895	15.531±2.066[a]	15.036±0.447[c]	8.401±0.679[b]	8.292±0.847[b]	15.586±0.885[a]

Data are presented as the mean ± SD. [a]P<0.05 compared with normal group; [b]P<0.05 compared with model group; [c]P>0.05 compared with model group. TIMP, tissue inhibitor of metalloproteinase; si, small interfering.

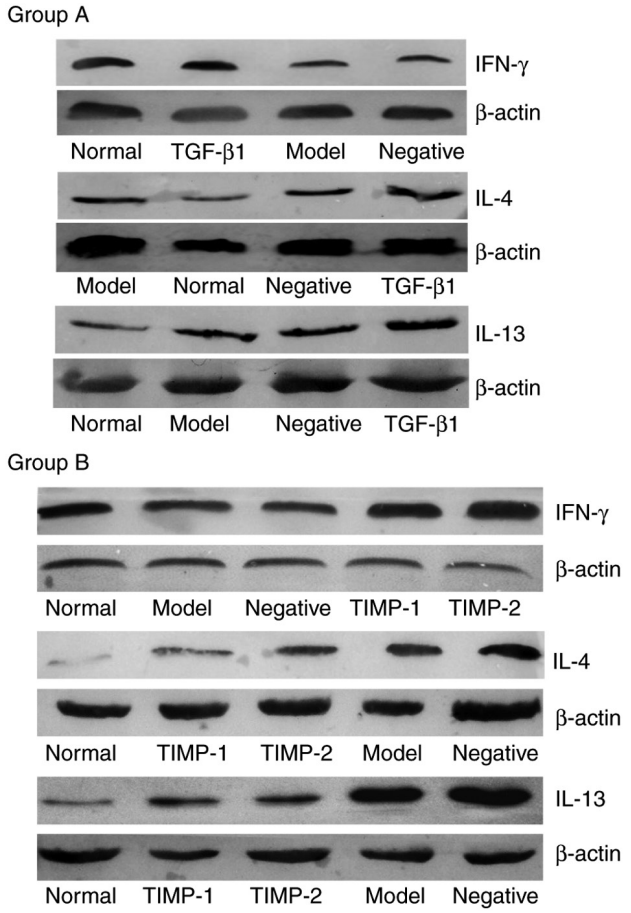

Figure 3. Expression levels of IFN-γ, IL-4 and IL-13 detected by western blotting. Band intensities were measured, and protein signals were normalized to β-actin levels. Group A, TGF-β1 siRNA treatment group. Group B, TIMP-1 and TIMP-2 siRNA treatment groups. TIMP, tissue inhibitor of metalloproteinase; si, small interfering.

and IL-13 in the TIMP-1 and TIMP-2 siRNA treatment groups were significantly decreased compared with those of the model group. There was no difference in the expression levels of IFN-γ, IL-4 and IL-13 between the negative control group and the model group in either group A or B (Fig. 3; Table II).

Expression levels of IFN-γ, IL-4 and IL-13 detected using RT-qPCR. Compared with those of the normal group, the expression levels of IL-4 and IL-13 were higher and the level of IFN-γ was lower in the model group. Group A and group B exhibited the same results. It was observed that in the TGF-β1 siRNA treatment group (group A), the expression levels of IL-4 and IL-13 were similar to that in the model group; by contrast, the expression of IFN-γ was significantly increased. In the TIMP-1 and TIMP-2 siRNA treatment groups (group B), the expression levels of IL-4 and IL-13 were significantly decreased compared with those in the model group, and the expression of IFN-γ was significantly increased. There was no significant difference in the expression levels of IFN-γ, IL-4 and IL-13 between the negative control group and the model group of either group A or B (Fig. 4).

Discussion

Hepatic fibrosis is a pathological process, through which numerous types of chronic liver diseases, including chronic viral hepatitis, alcoholic hepatitis and drug induced hepatitis eventually develop into cirrhosis. Hepatic fibrosis causes lobular alterations and the formation of false lobules and nodules (1). Hepatic fibrosis can develop into cirrhosis via various mechanisms, causing serious adverse consequences, such as portal hypertension and liver failure (9). Research on the mechanism of hepatic fibrosis is being currently conducted; increasing experimental and clinical evidence suggests that cytokines serve an important role in the regeneration of liver cells, the activation of hepatic stellate cells and the synthesis and degradation of ECM (2,3,6). Among them, the role of Th1/Th2 immune imbalance in hepatic fibrosis is also becoming increasingly investigated (10).

IFN-γ is an important Th1 factor, as it promotes the differentiation of Th1 cells and inhibits the differentiation of Th2 cells (11). IFN-γ can inhibit the activation and proliferation of myofibroblasts and promote their apoptosis, thereby suppressing the production of ECM (12). In addition, IFN-γ can promote Th1-type and inhibit Th2-type cytokine responses, thereby inhibiting the proliferation of fibroblasts and the deposition of ECM (13). The results of a previous study have demonstrated that IFN-γ also promotes the apoptosis and directly inhibits the activation of HSCs, thereby reducing the synthesis and secretion of collagen and other ECM factors (14). Li *et al* (15) used the Th1 cytokine IFN-γ to treat rats with hepatic fibrosis, which was induced via thioacetamide injection, and indicated that collagen deposition in the liver was significantly reduced after IFN-γ treatment.

IL-4 is an important Th2 factor, and it can promote the activation of B lymphocytes and the differentiation of $CD4^+$ T

Table II. Expression levels of IFN-γ, IL-4 and IL-13 detected via western blotting.

Cytokine	Normal group	Model group	TGF-β_1 siRNA treatment group	TIMP-1 siRNA treatment group	TIMP-2 siRNA treatment group	Negative control group
IFN-γ	0.835±0.189	0.562±0.133[a]	0.746±0.091[b]	0.785±0.024[b]	0.853±0.027[b]	0.410±0.016[a]
IL-4	0.354±0.018	0.871±0.003[a]	0.798±0.020[c]	0.390±0.018[b]	0.473±0.120[b]	0.798±0.090[a]
IL-13	0.461±0.128	0.923±0.156[a]	0.819±0.343[c]	0.392±0.039[b]	0.426±0.007[b]	0.819±0.091[a]

Data are presented as the mean ± SD. [a]P<0.05 compared with normal group; [b]P<0.05 compared with model group; [c]P>0.05 compared with model group. TIMP, tissue inhibitor of metalloproteinase; si, small interfering.

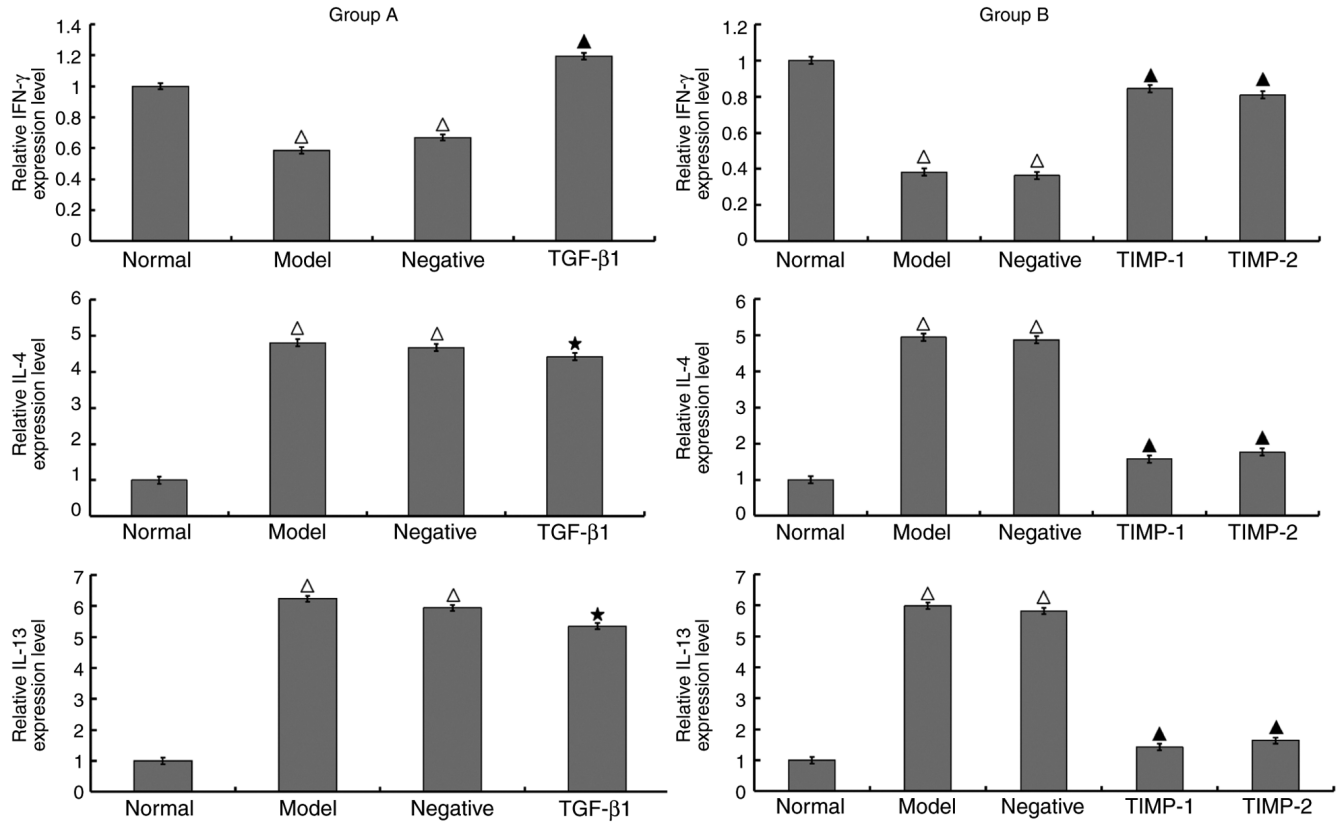

Figure 4. mRNA expression levels of IFN-γ, IL-4 and IL-13 in rat liver. Group A, TGF-β1 siRNA treatment group. Group B, TIMP-1 and TIMP-2 siRNA treatment groups. All expression data were normalized to GAPDH expression. [Δ]P<0.05 compared with normal group; [▲]P<0.05 compared with model group; [*]P>0.05 compared with model group. TIMP, tissue inhibitor of metalloproteinase; si, small interfering.

cells into Th2 cells (16). Atsukawa *et al* (17) demonstrated that IL-4 could induce liver Kupffer cells into multinucleated giant cells and stimulate the proliferation of HSCs. *In vitro* experiments by Bergeron *et al* (18) indicated that IL-4 could promote the expression of type I collagen and reduce the expression of matrix metalloproteinase 2, leading to hepatic fibrosis. IL-13 is mainly expressed by Th2 cells and is a potent profibrotic factor. Weng *et al* (19) confirmed that the cytokines IL-4 and IL-13 are important drivers of hepatic fibrosis progression, and IL-13 and IL-4 share a common IL-4 receptor subunit α-STAT6 pathway and exhibit similar biological functions. Coutinho *et al* (20) revealed that IL4, IL-5 and IL-13 serve important independent roles in liver cirrhosis caused by schistosomiasis, and suggested that the reason that these patients were predominantly male may also be associated with Th2 cytokines. IL-4 and IL-13 stimulate collagen synthesis via the TGF-β1-Smad3 pathway, as it has been indicated that IL-4 and IL-13 can increase the expression of matrix metalloproteinases, separate the latency-associated peptide-TGF-β1 complex (inactive TGF-β1) and activate TGF-β1 indirectly (21). IL4 and IL13 can also independently stimulate the synthesis of collagen, and IL-4 receptor was found in numerous subtypes of mice and human fibroblasts (22). *In vitro* studies have indicated that IL-4 and IL-13 can stimulate ECM synthesis from fibroblasts, such as collagen type I, collagen type III and fibrinogen (23,24).

The results of the present study suggested that the expression levels of IL-4 and IL-13 in rats with hepatic fibrosis induced by CCl_4 were significantly increased compared with those in normal rats, and the expression of IFN-γ was decreased.

This finding is consistent with the results of previous studies, such as in hepatic fibrosis induced by schistosomiasis (11,20). Treatment with TGF-β1 siRNA reduced the degree of hepatic fibrosis, but the expression levels of IL-4 and IL-13 in the liver were not significantly reduced compared with those of the model group. To hypothesize, the first possible reason may be that IL-4, IL-13 and TGF-β1 are effective anti-inflammatory factors. When rats with hepatic fibrosis were injected with TGF-β1 siRNA to block or reduce the expression of TGF-β1, rat liver was still subjected to damage by CCl_4, suggesting that hepatic inflammation was persistent. To counteract persistent inflammation, the body may secrete increased levels of compensatory IL-4 and IL-13. To further hypothesize, the second possible explanation may be that as the TGF-β1-Smad3 pathway is one of the mechanisms via which IL-4 and IL-13 stimulate collagen synthesis, when the expression of TGF-β1 is interfered with, the body may express more IL-4 and IL-13 due to a feedback mechanism. Treatment with TIMP-1 siRNA and TIMP-2 siRNA reduced the degree of hepatic fibrosis and markedly reduced the expression levels of IL-4 and IL-13 in the liver compared with those of the model group. Although TGF-β1, TIMP-1 and TIMP-2 are important targets for antifibrotic treatment, it is hypothesized that TGF-β1 was not an ideal antifibrotic target, as indicated by the expression of Th2 cytokines. By contrast, TIMP-1 and TIMP-2 were more suitable targets for treating hepatic fibrosis. Notably, the alterations in the aforementioned inflammatory factors indicated that Kupffer cells are a key component of liver fibrosis, which is worthy of further study. In addition, to the best of our knowledge, there are no drugs targeting TGF-β1, TIMP-1 or TIMP-2 in the treatment of liver fibrosis, and the therapeutic effect of antifibrotic drugs may not be ideal. The treatment of liver fibrosis is still a key problem to be solved in the clinical practice. Therefore, the current study can provide novel insights for the development of antifibrotic drugs.

Acknowledgements

Not applicable.

Funding

The present study was sponsored by a grant from The National Natural Science Foundation of China (grant no. 81270503).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

YX and KQ were responsible for the conception of data, data collection and drafting the manuscript. YX, KQ, YS, LX and XS all participated in the analysis and interpretation of data. In addition, XS also supervised and funded this study, made substantial contributions to conception and design and reviewed and modified the manuscript. YX and KQ confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All animals received humane care that was approved by the Institutional Animal Care and Ethic Committee the Second Affiliated Hospital of Chongqing Medical University (Chongqing, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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